The Occurrence of Sarcotaces in Canada

By E. Kuitunen-Ekbaum, Ph.D.
School of Hygiene, University of Toronto

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ABSTRACT

A parasitic copepod, encysted in the abdominal cavity and in the muscles of the red snapper, *Sebastodes ruberrimus*, caught off the Pacific Coast of Canada, has been identified as *Sarcotaces arcticus*. Each cyst contained one female and one male copepod and had an opening through the skin of the fish by which, no doubt, the nauplius larvae are discharged.

OCCURRENCE

The red snapper, *Sebastodes ruberrimus*, was the only fish in which the parasite was found, although it cannot be stated that *Sarcotaces* in British Columbian waters is confined to this host only. The fish were caught off the east coast of Vancouver Island by fishermen while fishing for lingcod. The infected fish were obtained from the United Fishermen's Co-operative Society, Nanaimo, during the month of August, 1946. According to information obtained from fishermen, they had been long aware of the parasitism in the red snapper and had noticed that the infection was more common during the winter than during the summer months. Some specimens of *Sarcotaces* had been collected and isolated from the red snapper between 1940 and 1945 by Dr. J. L. Hart, Pacific Biological Station, but the parasite was not identified.

Although in one fish the cyst appeared as a swelling over the operculum, and in another it could be seen in the muscles under the left pectoral fin, the majority of the infected fish appeared normal, the occurrence of parasitism being indicated by the black fluid oozing among the fish lying in fish bins. The most common location of the cysts was the abdominal cavity near the anus. In each infected fish there were usually one to three cysts with living parasites and adjacent to these there were frequently clusters of small atrophied cysts.

The *cyst* is gray in colour, smooth, firm and pyriform in shape (fig. 1, A). It hangs loosely in the body cavity or in the tissues of the host, except for its attachment near the surface of the skin. The cyst wall is so thin at the broad distal end that the parasite can be seen through it; it gradually thickens toward the tapered end of the cyst where it has grown to the adjacent organs and tissues.

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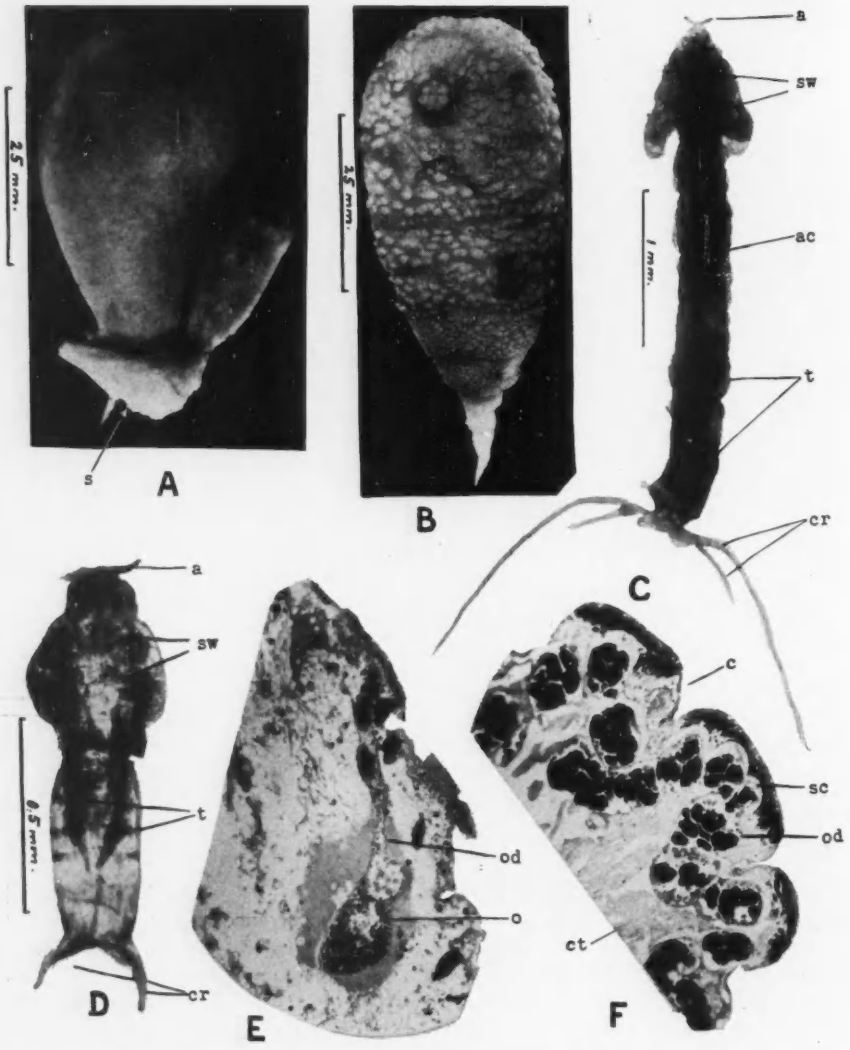

FIGURE 1. *Sarcotaces arcticus:* A—cyst with live parasites (left); caudal end of the female is seen protruded through the skin of the fish; small atrophied cyst (right); B—female, ventral view; C—male; D—juvenile male; E—section of ovary; F—section through verrucae; a—first antennae; ac—alimentary cavity; c—cuticula; cr—caudal rami; ct—connective tissue; o—ovary; od—oviduct, showing egg-cells and large masses of yolk cells; s—skin of the fish; sc—subcuticular layer of cells in verrucae; sw—swimming feet; t—testes.

At the small end of the cyst is the aperture leading to the surface and through this opening the caudal end of the female parasite may be seen protruding.

In some cases two or more cysts were fused together at their small ends beneath the skin of the fish but the cyst cavities were separate; clustered together with these cysts containing living parasites were shapeless remains of others, filled with a black, hard mass. These atrophied cysts were considered to be the end stage of the life cycle; parasites had atrophied and shrunken within the cysts after the discharge of the larvae. In some cysts the parasites had apparently failed to keep the communication with the exterior; the contents of such rudimentary cysts contained countless dead, but fully developed nauplii encased within the cyst wall.

The cyst wall is composed of layers of dense connective tissue and muscle fibres, with a rich supply of blood vessels. When the cyst is cut open there is a small amount of colourless fluid between the parasite and the cyst wall, barely enough to moisten the surface, since the parasite fills the cavity completely. Each cyst contained a large female and one small male; in some instances one female and two males were found in one cyst. Once the male parasite was observed it was possible to detect its presence within the cyst where it appeared through the cyst wall as a small dark rod.

The female isolated from the cyst closely resembles the pyriform cyst in size and shape (fig. 1, B). The parasite is greyish yellow except where the black contents of the alimentary cavity show through the body wall. The anterior end is broadly rounded, the caudal end tapered. The specimens measured from 5 to 70 mm. in length and 1.3 to 30 mm. in width at the broadest part of the body near the anterior end; the average sizes being 40 mm. long and 25 mm. wide. The body is verrucose without apparent limbs or antennae. The verrucae are larger at the anterior end of the body and gradually diminish in size towards the caudal end. The body of the parasite is composed of seven segments which represent the cephalothorax, followed by three abdominal segments; the latter are smooth, firm, composed of thick cuticle and with very few verrucae. On the ventral surface near the anterior end of the cephalothorax is a shallow depression in which is included a rosette of five to six radiating papillae and near the centre of it is another minute rosette of papillae which surround the oral opening. When the oral area is isolated and cleared in clove-oil, it shows two or three pairs of small rudimentary appendages which are probably the mandibles and maxillae. The oral opening is very small, encircled with a ring of striated muscles, from which point one pair of muscle bands is directed anteriorly and another posteriorly. The buccal cavity leads into a short oesophagus which opens into a wide and spacious alimentary cavity; the latter occupies almost the entire coelom. The narrow space between it and the body wall is occupied by loose connective tissue and bands of striated muscle. The alimentary cavity terminates blindly even in the youngest specimen (5 mm. long) found; the hind gut and the anus having apparently atrophied at an early stage of development. The digestive epithelium of the alimentary cavity is composed of high cylindrical cells; in the distal portion the epithelium is higher and thrown into folds. The

alimentary cavity is filled with a black suspension which is digested blood from the host; in the suspension some blood cells of the fish were usually found. The body is provided with heavy bands of striated muscles which originate from the dorsal surface at the level of the mouth depression and extend posteriorly to the first abdominal segment. A small pear-shaped ovary is situated on either side of the anterior portion near the dorsal surface of the cephalothorax (fig. 1, E). The oviducts are simple outgrowths from the anterior portion of the ovaries; their ramifications extend into the subcuticular space throughout the cephalothorax. They form a dense network and open to the exterior on the ventral side of the seventh segment. In the material available for the present study these openings were visible only in serial sections. The convolutions of the oviducts are filled with egg-cells in various stages of development, a thin membrane surrounding the developing ova and yolk cells (fig. 1, C). The body wall is composed of a thin cuticle underneath which in the area of verrucae is a layer of high columnar cells; between the warts this layer is discontinuous so that in the valleys the convolutions of the oviducts are covered only with a thin cuticular layer.

When isolating the female copepod, masses of nauplius larvae were found within the cyst on the surface and especially between the verrucae and in the fluid between the parasite and cyst-wall. The nauplius is oval in shape, about 0.2 mm. long and 0.12 mm. wide, and provided with three pairs of appendages. The caudal end of the body is rounded and bears two setae (fig. 2, A).

The male is elongate, slender, flattened dorsoventrally; the specimens found measured from 1 to 2.8 mm. in length and 0.2 to 0.3 mm. in width (fig. 1, C, D). The anterior end terminates with a small subquadrangular head separated from the thorax by a more or less clearly defined constriction. The head bears two pairs of antennae and two pairs of mouth parts. The first antennae, attached to the frontal margin of the head, are composed of five segments and tipped with a tuft of fine setae. The second antennae are small, attached to the ventral surface, posterior to the bases of the first antennae; each is composed of three segments and tipped with two short and stout claws. The oral appendages are probably mandibles and a pair of maxillae. The anterior portion of the thorax is expanded laterally and this area bears two pairs of biramose swimming feet (fig. 2, J, H). Both pairs of appendages are about equal in size, but in the first pair the endopod terminates in two spines and the exopod in four; in the second pair both the endopod and exopod terminate in three spines. The segmentation of the body is indistinct in senile individuals. In the only juvenile specimen found which was 1 mm. long, the thorax was composed of five segments; the first two thoracic segments apparently being fused. The abdomen is represented by the last segment which bears the caudral rami; the latter are short and conical in the juvenile specimen and replaced by two pairs of appendages at the later stages; in senescent individuals one pair of appendages is found and in more advanced stages of atrophy the caudal end is without appendages (fig. 2, J, K, L, M).

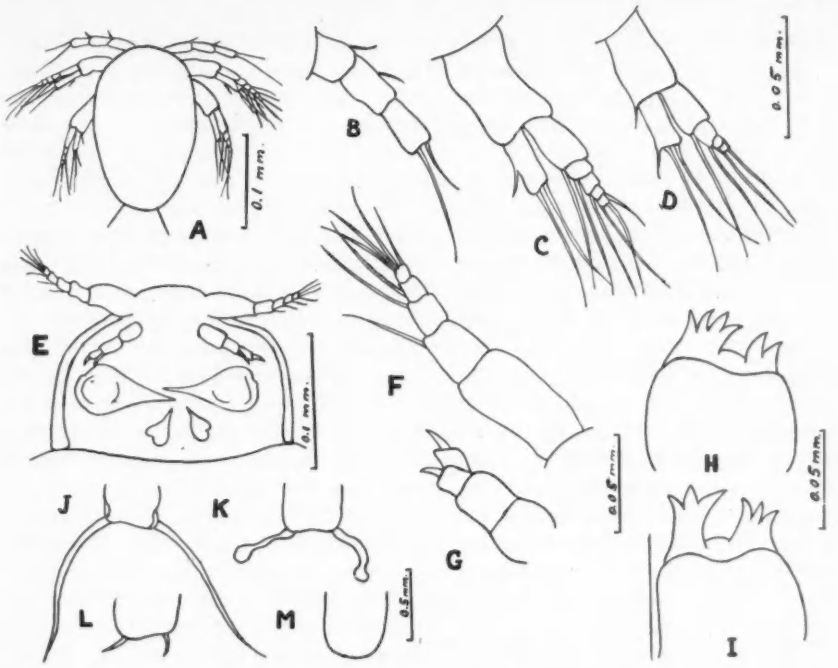

FIGURE 2. *Sarcotaces arcticus:* A—nauplius; B, C—first and second antennae; D—mandible of nauplius; E—head of male; F, G—first and second antennae of male; H, I—first and second swimming feet of male; J, K, L, M—caudal ends, showing various stages of atrophy.

The small mouth leads into a short oesophagus which opens into a wide alimentary cavity. The latter, as in the female, terminates blindly and is filled with a black suspension. A rudimentary intestine is visible in the juvenile individual, extending like a fine thread from the alimentary cavity to the end of the abdomen.

The elongate testes lie one at each side of the body, dorsal to the alimentary cavity, and extend anteriorly from the posterior part of the thoracic region. Vasa deferentia are continuous from the testes, extending anteriorly, and forming convolutions in the lateral lobes of the thorax; thence they turn back and enter into a pair of seminal vesicles; the genital openings, which are on the ventrolateral side of the genital segment, are indistinct in senescent individuals. Through various retrogressive changes during the life cycle the senescent individuals lose much of their identity with the younger forms; the caudal rami are replaced by two pairs of appendages which finally disappear; the testes become rounded, much enlarged, and located at the posterior end of the body; the head acquires a more or less triangular shape and is finally shifted below the anterior margin of the thorax; occasional irregularly spaced spines appear at the posterior

portion of the body; the lateral thoracic expansions become indistinct following reproductive period; the digestive cavity and testes are still present in individuals in advanced state of senescence. The mouth-parts, antennae and swimming feet are similar in size and shape in the young as well as the senile forms.

DISCUSSION

The present report is the first record of the occurrence of *Sarcotaces* in Canadian waters and in a new host. The genus has been hitherto described from distant localities and from unrelated hosts and every species described thus far has been restricted to a single species of the fish host. The first specimen, one female, was collected by Goës from an *Acanthurus* species caught in the West Indies in 1866 and described by Olsson as *Sarcotaces verrucosus* in 1872. Collett described the second species, *S. arcticus*, in 1874 from *Molva abyssorum* which were caught in arctic Norway at Oxfjord, Finmark. The copepod was differentiated from *S. verrucosus* by the larger size and by the absence of oral appendages. No male parasites were found.

Hjort, studying specimens received from Collett, published a report on morphology and life cycle of *Sarcotaces arcticus* in 1895. He speculated that after the period of growth in the tissues of the host the parsite is cut off from food by the development of a cyst-wall and must subsist further on the blood it has stored in the alimentary cavity; when the parasite has ripened it degenerates; the cyst wall disintegrates; an opening to the surface of the fish is formed and the nauplius larvae escape into the sea. He described the nauplius as having no setae or horns. No male parasites were found on most careful search of the sediment taken from the cysts and the muscles of the fish. He suggested that *Sarcotaces* might not be a copepod, but might represent a new type of a specially modified parasitic cirripede.

Calman in his study of Crustacea in 1909 included the genus in the *Appendix to Rhizocephala;* he admitted that the systematic position of *Sarcotaces* was obscure and further investigation would determine what relation, if any, the genus bears to the true *Rhizocephala*.

Komai described the third species *Sarcotaces pacificus* in 1924. He found 14 parasites in the subcutanepus tissues of various parts of the body in a single specimen of an *Antennarius* sp. in Japan. Each parasite was enclosed in a cyst with no communication with the surface. The copepod was differentiated from *Sarcotaces verrucosus* by the absence of setae around the oral opening and from *Sarcotaces arcticus* by smaller size, 5 to 15 mm. long, and by presence of oral appendages. He described and figured both testes and ovaries in each individual, but in spite of the hermaphroditic nature of the parasite he suggested that *Sarcotaces* was not a cirripede but a copepod.

Dollfus reported the occurrence of *Sarcotaces verrucosus* in an *Iridio radiatus* in 1928. The infected fish, caught at Martinique, had about ten cysts on its sides. Each cyst contained a female copepod which he found to be identical with the specimen described by Olsson. The parasite had a pair of short parabuccal

appendages and a number of chitinous lamellae directed radially around the oral opening.

Aitken reported *Sarcotaces arcticus* from *Molva abyssorum* in Aberdeen in 1942. He examined 26 cysts, each of which contained one adult female, and in 19 of them he found the hitherto unknown male of *Sarcotaces*. He gave a short description and figures of these specimens, which were up to 3 mm. long, with four pairs of appendages on the head and two pairs of biramous appendages on the trunk. The telson was provided with caudal furca variable in size. The alimentary cavity ended blindly; behind it were two masses of cells suggesting testes but the material was too poorly preserved to give a definite answer.

It was possible to examine a female specimen of *S. verrucosus* loaned by Dr. R. Ph. Dollfus and several specimens of *S. arcticus*, females and males, sent by Dr. A. Aitken. Both species were found to be identical with the specimens from British Columbia. Unfortunately it was not possible to dissect the museum specimen of the female *Sarcotaces* from Martinique and compare the mouth parts with those of the specimens in the present collection. Dollfus did not state that the copepod from *Iridio radiatus* possessed a crown of setae around the oral opening, but indicated a number of chitinous lamellae directed radially around the mouth. It was noted with the females in the present series and those, donated by Aitken, that when the oral opening with the perioral area was isolated and cleared in clove-oil, the striated muscles encircling the oral opening simulated a fringe of delicate radiating setae. If this were the case with the Olsson's specimen, the females of all three species would appear to be identical. If the male of *S. verrucosus* should be discovered and found to be similar to that of *S. arcticus* the latter would become synonymous with *S. verrucosus*.

The male specimens of *Sarcotaces articus* in Dr. Aitken's collection are identical with senescent stages found among the males in British Columbia.

Komai differentiated *Sarcotaces pacificus* from *S. arcticus* by the smaller size and the presence of oral appendages. The present studies have shown that the female of *Sarcotaces arcticus* possesses vestiges of oral appendages and the range in size of the females includes the dimensions of both, *Sarcotaces arcticus* and *S. pacificus*. Komai isolated fourteen parasites from one fish about 15 cm.; it is unlikely that the parasites in a fish of that size would reach the dimensions found in the red snapper 40 to 60 cm. long. Unfortunately Komai failed to find the male parasite which would have provided further confirmation in the specific identity of *Sarcotaces* found in Japan. According to his description and figures, the female and the nauplius are similar to those from British Columbia, except in the matter of his description of ovaries as testes and the oviducts as ovaries. Since the mature female of *Sarcotaces* has become degenerated because of its parasitic habit, only the finding of the male would definitely settle the question of whether or not *Sarcotaces pacificus* is a valid species.

The conjecture of Hjort that the parasite is unable to take food during the period of encystment, does not apply to the specimens from British Columbia. Blood cells were found in the black suspension in the digestive cavity, especially

in younger parasites. Few blood cells or none were found in the alimentary cavity of ripe females and of senescent males.

Much of the life cycle, especially the stages between the nauplius and mature parasites is largely unknown. Further investigation of early stages and especially of male forms will show whether *S. arcticus* and *S. pacificus* are separate species or synonymous with *Sarcotaces verrucosus* Olsson.

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Amino Acid Composition of Fishery Products

By Catherine P. Deas and H. L. A. Tarr
Pacific Fisheries Experimental Station
Vancouver, B.C.

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ABSTRACT

Fish flesh and certain waste materials were hydrolysed by tryptic enzymes of fish pyloric caeca. Fractionation of the resulting hydrolysates showed that they contained largely peptone, sub-peptone and residual (small peptides and amino acids) nitrogen, and little or no protein or proteose nitrogen. Fish flesh, milts, roes, meal, stickwater and a muscle myosin preparation were extracted to remove the fat, then dried and hydrolysed with acid or alkali. The essential amino acids arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, tryptophane and tyrosine were determined in these enzyme-, acid- and alkali-hydrolysed materials by microbiological methods. The results have been summarized.

Fish and fish products form an extremely important protein item of both human and livestock diets, yet the amount of detailed information regarding the amino acid distribution in these foods is, in comparison with that of many other protein foods, rather limited. This is apparent when reviews such as those of Block and Bolling (1945) and of Block and Mitchell (1946) are consulted. It is for this reason that the present work, already reported briefly elsewhere (Deas and Tarr 1946, 1947), was initiated.

ENZYME HYDROLYSIS

Method Used

Hydrolysis was effected by the action of crude tryptic enzyme preparations prepared by blending washed fish pyloric caeca in a Waring Blendor, the following general conditions being observed. One-hundred-gram quantities of blended fish material were placed in 500-ml. conical flasks together with a given weight of enzyme preparation (or commercial pancreatin), 1N NaOH solution to adjust the pH to 9.0, and water to bring the final weight to 400 g. In general this initial pH value maintained the sample within the range of activity of such tryptic enzymes (Johnston 1937). The preparations were covered with a layer of toluene, the flasks stoppered, and incubated in a water thermostat at 40°C. with frequent shaking, particularly during the first few hours, since even with finely divided material there is considerable danger of bacterial putrefaction. The comparative rate of hydrolysis was determined by the simplified formol titration method of Brown (1923) and pH values with a Beckman instrument.

In one experiment of an exploratory series flesh of "red cod" (*Sebastodes* sp.) was hydrolysed with different amounts of pyloric caeca, and also with com-

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mercial dry pancreatin (Coleman and Bell). The results indicated that 10 or 15 g. of the pyloric caeca was about as effective for hydrolysis as 2 g. of pancreatin. Hydrolysis was rapid at first and was about complete after 16 days, the final pH values of the suspensions being between 7.2 and 7.8.

Source and Preparation of Samples

red cod flesh.

Skinned fillets were blended and 100-g. quantities were hydrolysed for 16 days with 15 g. of red cod enzyme preparation under the conditions described above. The contents of the flasks were combined, filtered, and the oily layer separated. The clear aqueous filtrate was adjusted to pH 6.0 with dilute HCl and stored under toluene at room temperature.

sockeye (*Oncorhynchus kisutch*) waste.

One-hundred-gram quantities of small flesh particles collected from cannery filling machines were steamed 10 minutes at 100°C. in the flasks to be used for hydrolysis. The containers were then stoppered and stored several days at 0°C., until a satisfactory rock cod (*Sebastodes* sp.) enzyme preparation was available. The samples were then hydrolysed and stored under conditions similar to those used for red cod flesh.

herring (*Clupea pallasii*) flesh.

Gibbed herring were minced and then hydrolysed with a herring enzyme preparation under the above described conditions.

dogfish (*Squalus suckleyi*) waste water.

The alkaline waste water from a dogfish liver oil centrifugal extraction process was adjusted to pH 9.0 with dilute HCl, and 300-g. portions were hydrolysed with 15 g. of herring enzyme under the usual conditions. Since the colloidal nature of this material prevented filtration, even after enzyme hydrolysis, it was stored as above and used without clarification.

black cod (*Anoplopomá fimbria*) waste water.

This alkaline material was obtained from a black cod visceral oil extraction process. It was hydrolysed as was the dogfish liver waste water, and filtered before storage.

Precipitates formed gradually in samples other than the "waste waters", and these were assumed to consist of tyrosine peptides, tyrosine or cystine. Before using the hydrolysates for analysis the precipitates were separated, warmed with dilute HCl until solution occurred, and returned to the main body of solution.

Nitrogen in Hydrolysates

The approximate degree of protein degradation in the above five preparations was determined by the method of Wasteneys and Borsook (1924). The results (table I) showed that, with the exception of the dogfish liver waste water, the proteins had been broken down to the smaller residues. Protein and proteose nitrogen were almost, or entirely, absent. Peptone nitrogen made up 20% to 25%, sub-peptone nitrogen 21.5% to 54.2% and residual nitrogen (small peptides and amino acids) 22.5% to 58.4% of the total nitrogen. The dogfish liver waste water contained a considerable quantity of both protein and proteose nitrogen. Evidently the material contained difficultly hydrolysable proteins such as collagen, elastin or heat- and alkali-altered proteins, which were not easily hydrolysed by the caecal enzymes.

Table I. Nitrogen distribution in enzyme-hydrolysed fish products.

Material	Nitrogen (%)	Dry wt. (%)	Protein (Nx6.25) (% of dry wt.)	Per cent total N present as:				
				Protein	Proteose	Peptone	Sub-peptone	Residual
Red cod flesh.....	0.89	6.3	88.3	0.0	0.0	20.0	21.5	58.4
Sockeye salmon waste.....	1.08	7.4	91.0	0.5	0.1	25.2	39.7	34.3
Herring flesh.....	1.4	10.3	83.5	0.7	0.0	20.6	44.3	34.3
Dogfish liver waste water.....	0.57	5.2	67.8	3.3	5.8	7.4	52.5	31.0
Black cod waste water.....	0.86	9.6	56.3	0.0	0.0	23.4	54.2	22.5

Amino Acids in Hydrolysates

The microbiological method described by Stokes and Gunness (1945) and Stokes, Gunness, Dwyer and Caswell (1945) for determination of essential amino acids was followed. *Streptococcus faecalis* was employed for analysis of all amino acids except phenylalanine, for which *Lactobacillus delbruckii* was used. It was found that satisfactory analyses could be obtained when hydroxyproline and norleucine were omitted from the basal medium. The amino acids were assayed at five different levels, four replicates being run at each level (Wood 1946). Assay tubes were incubated for 2 days at 37°C., and the medium then titrated to pH 7.0 using bromthymol blue indicator.* Tests in which known amounts of amino acids were added to the hydrolysates that were being assayed showed that recovery of from 92% to 101% of the theoretical amount could be obtained.

*In some of the present work, and in subsequent experiments which will be reported later, the speed and accuracy of these assays were greatly facilitated by use of automatic dispensing and titrating units obtained from Dr. Mott Cannon of the University of Wisconsin.

Values for arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, tryptophane and tyrosine were obtained for the enzymic hydrolysates. In addition, chiefly for comparative purposes, the tyrosine and tryptophane content of an alkaline hydrolysate of the enzymic digests was determined. Alkaline hydrolysis was effected by digesting 25 g. of the enzyme hydrolysates for 10 hours at 120°C. with 10 g. of 5N NaOH. The resulting solutions were neutralized with dilute HCl, the precipitates removed and washed, and the washings returned to the filtrate. The results of amino acid analyses are recorded in table II.

TABLE II. Amino acid content of enzyme-hydrolysed fish products (g. amino acid per 16 g. of nitrogen).

Material hydrolysed	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Valine	Tryptophane		Tyrosine	
										Enzyme alone	Enzyme followed by alkali	Enzyme alone	Enzyme followed by alkali
Red cod flesh	5.5	1.2	7.7	10.5	6.6	3.0	4.1	4.8	5.3	1.6	1.4	2.9	5.0
Sockeye salmon waste	0.6	1.0	6.7	10.3	5.0	2.9	4.1	4.4	5.9	1.5	1.4	1.7	2.8
Herring flesh	2.6	±	4.0	6.3	6.1	3.3	2.8	2.7	3.8	1.0	0.85	1.3	2.5
Dogfish liver oil waste water	1.3	0.9	3.3	5.7	4.1	1.3	2.7	2,2	3.2	1.1	0.87	1.8	2.8
Black cod waste water	1.5	0.5	4.5	5.7	2.8	4.8	2.8	3.5	5.2	1.4	0.97	0.8	1.3

The content of essential amino acids in red cod flesh, sockeye flesh (waste) and herring flesh varied somewhat. Sockeye flesh (waste) gave a low arginine value, while herring gave lower values for histidine, leucine, isoleucine, phenylalanine, threonine and valine than did either of the other two materials. The two waste water samples gave analyses which, in general, compared favourably with those obtained for acid-hydrolysed stickwater (*vide infra*).

Racemization of tryptophane is assumed to be complete in alkali-hydrolysed protein materials, thereby necessitating the multiplication of results by two to get the correct values. However, values for enzymic hydrolysates were slightly, though consistently, higher than those from the same digests which had been treated with alkali. This is in accordance with the work of Greenhut, Schweigert and Elvehjem (1946) in which it was shown that treatment with either acid or alkali rendered pure tryptophane less utilizable by bacteria. On the other hand, increased values were obtained for tyrosine after alkaline hydrolysis of the enzyme digests, and this suggests that the tyrosine in peptides is not completely available to *Streptococcus faecalis* under the conditions of these assays.

ACID AND ALKALI HYDROLYSIS

Source and Preparation of Samples

Milts of white spring salmon (*Oncorhynchus tschawytscha*), chum salmon (*Oncorhynchus keta*) and herring, and roes from white spring salmon and herring were blended separately. The samples were covered with acetone and permitted to stand overnight. This extraction was repeated three times, thus removing most of the water and fat and denaturing the proteins. Red cod flesh as used in the preparation of the above-described enzyme hydrolysates was minced and then dried in a thin layer for 24 hours at 100°C. For comparative purposes a portion of the same flesh containing the quantity of caecal enzyme normally employed in enzyme hydrolysis was similarly dried. A single sample of herring meal was obtained, and also one of stickwater from herring reduction. The latter was dried in vacuum.

The dried samples were continuously extracted (Soxhlet) for 18 hours with acetone, and were then ground finely and stored in vacuum over P_2O_5 until used. In addition, a sample of myosin was prepared from lingcod (*Ophiodon elongatus*) by the method of Greenstein and Edsall (1940). Probably this preparation was impure "actomyosin". (Szent-Györgyi, 1945). The myosin gel was dried by exposure to increasing concentrations of ethyl alcohol and finally with ethyl ether. The finely dried material was stored over P_2O_5 in vacuum.

The dried samples were hydrolysed with HCl by the method of Barton-Wright (1946). The hydrolysates were stored under toluene at 0°C. and were used for the determination of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine. For the assay of tryptophane and tyrosine 0.5-g. samples were hydrolysed with 10 ml. of 5N NaOH for 10 hours at 120°C. The cooled hydrolysates were adjusted to pH 6.8 with HCl, the precipitates removed, washed, and the washings returned to the filtrates.

AMINO ACIDS IN HYDROLYSATES

The hydrolysates were assayed for essential amino acids by the method to which reference has already been made. The results, together with certain values for similar materials which have been reported in the literature, are given in table III. It will be seen that the amino acid distribution in the three samples of milts examined was very similar, though that from herring gave somewhat higher arginine values. There was also a similar distribution of amino acids in the two samples of roes examined. However, the arginine, histidine, isoleucine, lysine, methionine, tyrosine and valine values in the roes were all somewhat lower than those obtained for "salmon egg protein" by Block and Mitchell (1946). Variations such as differences in species or sexual maturity could easily account for such discrepancies. Since the whole roe contained more arginine than that found by Young and Inman (1938) in salmon egg casings, it is assumed that the yolk protein of the egg contains considerable amounts of this amino acid. The distribution of essential amino acids in the sample of herring meal resembled that recorded for "fish meal" by Block and Bolling (1945) and for sardine meal by

TABLE III. Amino acid content of acid and alkali hydrolysed products (g. of amino acid per 16 g. of nitrogen).

Material hydrolysed	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Valine	Tryptophane	Tyrosine
Herring milt											
Ash = 9.1%											
Nitrogen = 1.66%.....	13.5	0.6	3.1	5.0	1.5	0.9	2.3	2.7	3.1	0.37	0.14
White spring salmon milt											
Ash = 9.46%											
Nitrogen = 1.59%.....	11.1	0.46	3.2	5.6	1.8	0.92	2.3	2.4	3.3	0.35	1.5
Chum salmon milt											
Ash = 9.04%											
Nitrogen = 1.49%.....	11.9	0.7	3.5	5.7	2.1	1.1	2.4	2.4	3.3	0.32	0.81
Herring roe											
Ash = 2.57%											
Nitrogen = 1.36%.....	4.8	1.2	7.7	11.3	1.8	1.8	4.8	5.9	6.5	1.1	3.2
White spring salmon roe											
Ash = 2.91%											
Nitrogen = 1.46%.....	5.9	1.4	8.2	11.1	1.8	1.9	6.3	4.7	7.5	0.7	3.0
Salmon egg protein											
(Block & Mitchell 1946).....	6.4	2.7	9.9	10.7	3.8	3.0	5.3	4.5	9.4	0.8	4.0
Herring meal											
Ash = 15.21%											
Nitrogen = 1.08%.....	*	0.61	6.6	10.1	4.4	2.7	4.4	4.4	5.7	1.0	3.2
Fish meal (Block & Bolling 1945a)..	5.9	2.4	4	10.0	5.7	3	4.8	5	4	1.2	2.8
Fish meal (Block & Mitchell 1946)..	7.4	2.4	6.0	7.1	7.8	3.5	4.5	4.5	5.8	1.3	4.4
Stickwater											
Dry wt. = 6.75%											
Nitrogen = 0.96%.....	3.7	0.5	1.6	3.9	4.1	0.7	1.8	2.2	2.1	1.3	4.1
Fish stickwater (Block & Bolling											
1945a; Block & Mitchell 1946)..	5.4	2.6	1	2	4.1	1.5	1.9	2.3	3	0.8	0.8
(Lassen & Bacon 1946).....	4.34	5.79	2.73	4.67	4.87	1.51	2.33	2.35	2.98	0.35	....
Red cod flesh											
Ash = 3.99%											
Nitrogen = 1.44%.....	4.3	1.6	6.8	11.4	14.4	2.6	4.4	5.1	5.0	0.1	0.4
Red cod plus caeca											
Ash = 4.66%											
Nitrogen = 1.44%.....	5.8	1.8	6.0	8.7	10.6	2.5	4.7	4.6	4.7	0.8	2.8
Herring flesh											
Ash = 6.86%											
Nitrogen = 1.50%.....	5.9	2.0	6.4	8.0	8.2	2.2	4.5	4.5	5.1	0.7	2.2
Fish flesh.....(Min.)	4.6	0.6	5.2	6.4	5.7	3.0	4.3	3.4	4.3	0.9	0.9
(Literature**).....(Max.)	11.2	2.3	6.0	7.1	14.5	3.8	5	4.5	5.8	2.2	4.7
Myosin											
Ash = 0.87%											
Nitrogen = 1.51%.....	4.8	2.7	7.7	10.2	15.0	2.3	4.4	5.8	6.0	0.9	2.7

*Consistent arginine values for herring meal could not be obtained.

**Arrizoni and Fischer 1943; Beach, Munks and Robinson 1943; Beveridge 1947; Block 1945, p. 125; Block and Bolling 1945; Block and Mitchell 1946; Pottinger and Baldwin 1939; Sharp 1936; Sharpenak, Balashova and Perzovskaya, 1934.

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(Insert at bottom of page 518)

In Table III, with the single exception of stickwater, all values for nitrogen should have the decimal point moved one place to the right to make the values 16.6% etc.

Block and Mitchell (1946). The tryptophane and tyrosine values were similar to those found for non-putrid herring meals by Ingvaldsen (1929). It will be seen that, in general, the essential amino acid content of the stickwater sample examined was similar to that given in a single available set of published figures, although a higher value was obtained for tyrosine and a lower value for arginine.

Widely divergent values for the amino acid composition of "fish" have been recorded in the literature, frequently without specific reference to species examined or type of material studied. This gives a doubtful significance to any comparison of values recorded in previous published work with those obtained for fish flesh in the present work. It can only be stated that figures obtained in the present work generally fell well within the ranges previously reported. Fish flesh appears to be characterized by its rather high lysine and leucine content and its rather low tryptophane content. There seems to be a need for a thorough survey of the amino acid composition of the flesh of different species of fish.

No very full published report on the amino acid distribution in fish muscle myosin appears to be available. Bailey (1944) examined myosin preparations from certain animals and from fish and concluded, with reference to amino acids in them, that "although we cannot maintain that all myosins are chemically identical the indications are that variations are not very large". The content of essential amino acids in the present lingcod muscle myosin was similar to that reported for rabbit myosin by Bailey (1937), the values for methionine, tryptophane and tyrosine agreeing closely with those reported by him for fish myosin.

Somewhat different analyses were given by dried red cod flesh hydrolysed alone and by that hydrolysed after pyloric caeca had been added in the proportions used in producing enzyme hydrolysates. Thus values for tyrosine, and to a lesser extent arginine, were higher, and those for leucine and lysine were lower, in the red cod flesh to which caecal enzyme preparation was added than in red cod flesh alone.

A comparison of certain of the results given in tables II and III shows that there are differences in the amino acid content as determined after enzyme and after acid hydrolysis. Thus lysine, arginine, phenylalanine and histidine values were lower when red cod flesh was hydrolysed with caecal enzyme than when the flesh plus enzyme mixture was dried and then subjected to acid hydrolysis. On the other hand, the enzymic hydrolysis gave higher values for methionine, valine, isoleucine and leucine. Alkaline hydrolysis yielded lower values for tyrosine and tryptophane than did enzyme hydrolysis alone, or enzyme hydrolysis followed by alkali hydrolysis.

SUMMARY

Aqueous suspensions of blended fish flesh adjusted initially to pH 9.0 were hydrolysed at 40°C. by tryptic enzymes of fish pyloric caeca. The clear hydrolysates obtained after removing residual solid material contained little or no protein or proteose, 20% to 25.2% of peptone, 21.5% to 44.3% of sub-peptone and 34.3% to 58.4% of small peptides and amino acids. Samples of waste waters

from dogfish liver and black cod visceral oil extraction processes were also subjected to enzyme hydrolysis. All these enzyme-hydrolysed protein materials were used without further hydrolysis for microbiological assay of the essential amino acids. For comparative purposes values for tyrosine and tryptophane were also obtained on enzyme-hydrolysed materials which had been further hydrolysed with alkali. In general the enzyme-hydrolysed flesh gave quite high values for all amino acids except histidine and tryptophane. Flesh samples hydrolysed by enzymes and then further hydrolysed by alkali gave high values for tyrosine, and rather low values for tryptophane.

Dried, acetone-extracted samples of fish flesh, milts, roes, herring meal and stickwater and also a sample of alcohol-dried fish muscle myosin, were hydrolysed with hydrochloric acid for determination of all the essential amino acids except tyrosine and tryptophane for which alkali hydrolysis was used. The values obtained for essential amino acids in fish flesh fell within the very wide limits which have been reported in the literature for this material. Fish flesh was found to have a rather high leucine and lysine, and a rather low tryptophane, content. Fish milts had a very high arginine content but were rather deficient in histidine, lysine, methionine, tyrosine, tryptophane and lysine. Fish roes gave a high value for leucine but had a much lower lysine content than did fish flesh.

The essential amino acid composition of stickwater and of herring meal was not unlike that given for similar materials in the literature. The values obtained for fish muscle myosin were similar to those previously reported in the literature for mammalian muscle myosin, and to the few available published figures for fish myosin.

Certain differences were observed in the values obtained for essential amino acids in identical materials which were hydrolysed by (a) enzymes and (b) by acid or alkali.

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Control of Rancidity in Fish Flesh III. Carbonyl Enediols as Antioxidants.

By H. L. A. Tarr and N. E. Cooke
Pacific Fisheries Experimental Station
Vancouver, B.C.

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ABSTRACT

The cyclic carbonyl enediols, reductic acid and *l*-ascorbic acid, strongly retarded fat oxidation in frozen minced red spring salmon or herring flesh stored at −10 or −20°C., the former being more effective than the latter. The acyclic carbonyl enediols and dihydroxymaleic acid with its sodium and ammonium salts and reductone were either ineffective or afforded only slight protection.

Previous experiments showed that *l*-ascorbic acid, when incorporated into fish flesh which was subsequently frozen, strongly retarded development of oxidative rancidity during storage (Tarr 1947, 1948). This finding has been substantiated in commercial trials in which fish fillets and steaks, or split fish, have been treated by dipping them for short periods into 1 or 2 percent ascorbic acid solutions prior to freezing and storing them (Bauernfeind, Smith, Batcher and Siemers, 1948; Stoloff, Puncochar and Crowther, 1948; Tressler 1947). Furthermore, d-iso ascorbic acid and 5, 6-diacetyl *l*-ascorbic acid have proven as effective as *l*-ascorbic acid as fish flesh antioxidants (Tarr 1948). The present work was undertaken with the object of determining whether certain of the known compounds possessing the unique chemical properties which characterize *l*-ascorbic acid were also capable of retarding rancidity development in fish, and also whether any of them were more effective than *l*-ascorbic acid. Brief reference to this work has already been made (Tarr and Cooke 1947).

EXPERIMENTAL

The occurrence, isolation and synthesis of chemical compounds which exhibit reducing properties similar to those of *l*-ascorbic acid has been adequately reviewed (Rosenberg 1942, Smith 1946). These all possess the carbonyl enediol group, $-C(OH)=C(OH)-C=O$, which is autoxidizable when the compounds are present in aqueous solution at ordinary temperatures. The rate of oxidation of certain of them may be accelerated by enzymes. Both cyclic and acyclic compounds of this type are known, and examples of both classes have been included in the present study.

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CYCLIC

l-Ascorbic acid. Hoffmann—La Roche Inc.; U.S.P.

Reductic (Reductinic) acid. Prepared from California rapid-set citrus pectin by the method of Reichstein and Oppenauer (1933). Methods of preparing this compound by total synthesis have been described recently (Koninklijke Industrieel Maatschappij voorheen Noury & van der Lande N.V. 1944, 1946).

ACYCLIC

Reductone. Prepared from glucose by the method of von Euler and Martius (1933).

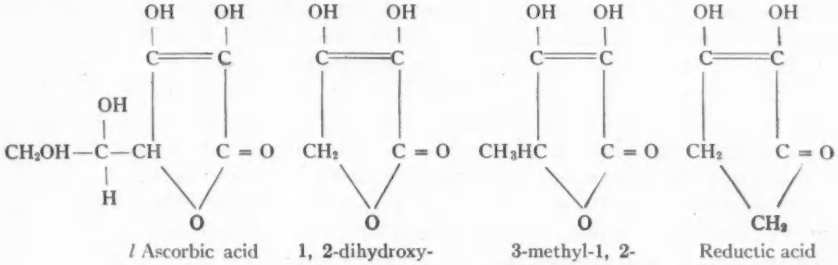
Dihydroxymaleic acid. Prepared by Nef's (1907) modification of Fenton's (1905) method.

Sodium dihydroxymaleate. Prepared by the method of Fenton (1894).

Ammonium dihydroxymaleate. Prepared by the method of Fenton (1894).

Since certain of the compounds used were somewhat difficult to prepare, only limited quantities of these were available for experimental work. Consequently tests have been restricted to small scale experiments using minced flesh. The methods of incorporating the antioxidants into the flesh, freezing and storing samples and determining peroxide values during storage were identical with those previously described (Tarr 1947). The antioxidants were incorporated in the minced flesh in the same molecular concentration, the following amounts being added: *l*-ascorbic acid, 0.04%; dihydroxymaleic acid; 0.0357%; sodium dihydroxymaleate, 0.0435%; ammonium dihydroxymaleate, 0.0415%; reductic acid, 0.0259%; and reductone, 0.02%. The purity of these compounds was established by one or all of the following methods: titration with standard iodine solution, titration with standard alkali solution or determination of the melting point. The titration values were within 99% of the theoretical amount.

Methods of synthesising cyclic hydroxytetronic acids having a lactone ring structure similar to that of *l*-ascorbic acid have been described (Micheel and Jung 1933, Micheel and Haarhoff 1940). So far these compounds have not been prepared for testing as antioxidants for fish flesh. The acid corresponding to reductone, namely enol tartonic aldehyde acid, cannot be prepared in stable form (Fischer, Baer and Nidecker 1937). The structure of the various carbonyl enediols referred to in this paper is given herewith for comparative purposes.

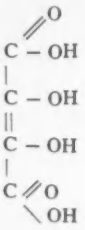


l Ascorbic acid

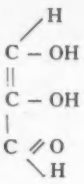
1, 2-dihydroxy-3-keto butyric acid lactone (1, 2-dihydroxy-tetrone)

3-methyl-1, 2-dihydroxy-4-keto butyric acid lactone (1, 2-dihydroxy-4 methyl-tetrone)

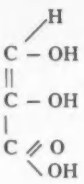
Reductic acid (Cyclo pentene-(2) - diol-(2, 3)-one(1))



Dihydroxymaleic acid



Reductone (Enol tartronic dialdehyde)



Enol tartronic aldehyde acid

RED SPRING SALMON (*Oncorhynchus tschawytscha*). The antioxidants listed in table I were incorporated into flesh obtained from a 6.5-kg. fish. In this ex-

TABLE I. Effect of reductic acid, *l*-ascorbic acid, reductone and dihydroxymaleic acid on development of rancidity in red spring salmon flesh.

Treatment	Peroxide value after days						
	− 10°C.			− 20°C.			
	18	41	94	94	187	363	453
Untreated	1.5	2.4	10.1	0.8	8.1	10.2	12.7
Reductic acid	0	0	0	0.0	0.0	0.1	0.6
l-Ascorbic acid	0	0	0	0.0	0.1	0.4	0.7
Reductone	0	0.2	2.8	0.2	1.0	4.1	5.2
Dihydroxymaleic acid	0.4	2.9	7.9	0.8	3.8	5.4	12.3

periment dihydroxymaleic acid, which is only very slightly soluble in water at ordinary temperatures, was dissolved in ethyl alcohol, the resulting solution being appropriately diluted with water so that a 1% solution of dihydroxymaleic acid in approximately 50% ethyl alcohol was obtained for incorporation in the minced flesh. The results of this experiment (table I) show that, with samples stored at − 10 and − 20°C., reductic acid was a slightly more effective antioxidant than *l*-ascorbic acid. In this experiment reductone exerted definite antioxidant activity while dihydroxymaleic acid was practically ineffective.

A further experiment was carried out with flesh obtained from an 11-kg. fish using the same antioxidants as were employed in the first experiment and, in addition, ammonium dihydroxymaleate. Both dihydroxymaleic acid and its ammonium salt were dissolved in warm water at not over 50°C. immediately prior to their inclusion in the flesh. The results of this experiment (table II) were rather similar to those of the preceding one. At both temperatures of storage reductic acid proved a somewhat more effective antioxidant than *l*-ascorbic acid, while dihydroxymaleic acid, ammonium dihydroxymaleate and reductone exerted no noticeable protective action.

TABLE II. Effect of reductic acid, *l*-ascorbic acid, reductone, dihydroxymaleic acid and ammonium dihydroxymaleate on development of rancidity in red spring salmon flesh.

Treatment	Peroxide value after days					
	− 10°C.			− 20°C.		
	18	69	95	69	186	361
Untreated	0.6	8.5	12.9	1.6	7.6	16.2
Reductic acid	0.0	0.7	1.7	0.1	0.8	2.3
l-Ascorbic acid	0.1	1.3	3.4	0.4	1.4	3.1
Dihydroxymaleic acid	2.0	8.4	9.1	2.8	9.1	15.0
Ammonium dihydroxymaleate	1.7	6.5	13.2	3.0	7.6	16.1

HERRING (*Clupea pallasii*). Four different antioxidants were incorporated in herring flesh, the dihydroxymaleic acid being added as an alcohol solution as in the first experiment with red spring salmon. The samples were stored at − 20°C. and were examined at intervals. The results (table III) show that reductic acid was a slightly more effective antioxidant than *l*-ascorbic acid. Reductone appeared to retard fat oxidation slightly, while dihydroxymaleic acid exerted a noticeable pro-oxidant effect.

TABLE III. Effect of reductic acid, *l*-ascorbic acid, reductone and dihydroxymaleic acid on development of rancidity in herring flesh.

Treatment	Peroxide value after days		
	15	72	238
Untreated	0.9	4.9	49.2
Reductic acid	0.0	0.4	10.5
l-Ascorbic acid	0.1	2.1	23.3
Reductone	0.3	3.9	37.1
Dihydroxymaleic acid	1.0	7.3	60.8

A further similar experiment was carried out in which, in addition to the antioxidants used in the preceding experiment, sodium dihydroxymaleate was also studied. Both the dihydroxymaleic acid and its sodium salt were dissolved

TABLE IV. Effect of reductic acid, *l*-ascorbic acid, reductone, dihydroxymaleic acid and sodium dihydroxymaleate on development of rancidity in herring flesh.

Treatment	Peroxide value after days		
	54	145	186
Untreated	4.9	8.6	14.8
Reductic acid	0.1	3.3	3.6
l-Ascorbic acid	0.5	3.7	4.2
Reductone	3.5	7.3	11.0
Dihydroxymaleic acid	5.1	10.9	11.8
Sodium dihydroxymaleate	3.4	6.2	8.0

in warm water immediately before incorporation in the flesh. The samples were stored at − 20°C. The results (table IV) show that, as in the first test with herring, reductic acid was somewhat more effective than *l*-ascorbic acid in retarding development of oxidative rancidity. Reductone, dihydroxymaleic acid and its sodium salt did not retard rancidity appreciably.

DISCUSSION

These experiments have shown that only the cyclic carbonyl enediols, reductic and *l*-ascorbic acids, were really effective antioxidants. The acyclic compounds were normally quite ineffective, though in one experiment with salmon reductone showed moderate activity. Previous experiments showed that an ice glaze containing incorporated *l*-ascorbic acid retarded fat oxidation in frozen salmon fillets much more effectively than did an ordinary water glaze (Tarr 1948). This observation has recently been extended, and it has been found that fat oxidation in whole frozen glazed herring is somewhat more strongly retarded by a glaze containing reductic acid than it is when l-ascorbic acid is used for this purpose, and that acyclic carbonyl enediols are either ineffective, or much less effective (Tarr and Kahn unpub.).

It has been assumed that *l*-ascorbic acid owes its effectiveness as an antioxidant for fish to its ability to remove oxygen from, or prevent its access to, the frozen tissues, thus preventing it from coming in contact with the fats. This contention is supported by the fact that *l*-ascorbic acid apparently undergoes slow oxidation in frozen fish flesh (Tarr 1948). It is possible that reductic acid is a more effective antioxidant for fish than *l*-ascorbic acid because in frozen medium it has an even greater affinity for oxygen than has the latter compound, and that the acyclic carbonyl enediols are normally ineffective because they do not readily oxidize under such conditions. Alternatively, the acyclic compounds may owe their relative ineffectiveness to the pro-oxidant activity of hydrogen peroxide which may be formed during their oxidation. It is known that hydrogen peroxide is formed during the autoxidation of dihydroxymaleic acid to the diketo compound at ordinary temperatures (Banga and Phillipot 1939). These suggestions can only be verified by further experiment.

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Nitrite-Reducing Bacteria on Cod Fillets

By C. H. Castell
Atlantic Fisheries Experimental Station, Halifax, N.S.

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ABSTRACT

Nitrite-reducing bacteria are present on fresh cod fillets; the proportion increases during storage and they shortly become the predominating flora.

Nitrite-reducing bacteria decrease the retarding action that small amounts of nitrite have on the bacterial reduction of trimethylamine oxide.

It has been shown by Tarr (1941, 1942) that sodium nitrite can be used advantageously as a preservative for fish, and that its bactericidal and bacteriostatic activities are closely related to the pH of the substrate, being inactive in the neutral or alkaline range. It was shown by Castell (1949) that concentrations of nitrite which do not inhibit bacterial growth, prolong the keeping quality of cod fillets by retarding the bacterial reduction of trimethylamine oxide. Dyer (1949) has subsequently pointed out that in nitrite-treated fish, the rapid reduction of trimethylamine oxide did not occur until there had been a significant reduction in the nitrite content. This would seem to indicate that with cod fillets, under ordinary conditions, the preservative value of the nitrite is dependent not only upon the initial quantity of nitrite used, but also upon the capacity of the microbial flora to reduce it.

Other than very general statements, little is known regarding the nitrite reducing activities of the microflora on fish or of the individual species. For that reason, more specific information on this point has been sought.

EXPERIMENTAL

Experimental Methods

For testing the ability of cultures to reduce nitrite, thirty to fifty parts per million of sodium nitrite were added to a broth consisting of 0.1% each of glucose, magnesium sulphate and dipotassium phosphate; 0.5% each of sodium chloride and Bacto peptone. This was adjusted to pH 6.8 before autoclaving. After inoculation, the cultures were incubated at 25°C. and examined at 5 and 7 days for evidence of growth and the presence of nitrite. Four drops each of the sulphanilamide and coupling reagents used in Dyer's (1946) colorimetric test for nitrite were added to one ml. of the solution to be tested. A complete absence of colour was recorded as positive. Evidence of bacterial growth and no reduction in the intensity of the colour reaction after 7 days was recorded as negative.

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EXPERIMENT 1.

Although certain cultures in our stock culture collection are not very closely associated with fish or fish spoilage, it was thought that it would be worth while testing all that were available in order to give as complete a picture as possible. The figures in brackets indicate the number of strains of each species that were tested:

Reduced Nitrite		*Did Not Reduce Nitrite*	
Escherichia coli	(6)	Micrococcus freudenreichii	(2)
" communior	(2)	" varians	(5)
Aerobacter aerogenes	(4)	" aureus	(5)
" cloaceae	(2)	" albus	(2)
Salmonella enteriditis	(2)	" citreus	(3)
Eberthella typhi	(2)	" aurantiacus	(2)
Serratia marcescens	(3)	" epidermidis	(5)
Bacillus subtilis	(4)	" roseus	(2)
" mycoides	(2)	" rubens	(4)
" mesentericus	(2)	" cinnabareus	(1)
Pseudomonas fluorescens	(2)	" conglomeratus	(1)
" putrefaciens	(8)	Sarcina lutea	(1)
Proteus vulgaris	(6)	Flavobacterium proteus	(2)
		" marinum	(2)
		" solare	(2)

EXPERIMENT 2.

A group of 299 cultures were isolated from cod fillets after storage of several days at 3°C. These were identified as far as genera and tested for nitrite reduction with the following result:

Genera	*Number of Cultures Not Reducing Nitrite*	*Number of Cultures Reducing Nitrite*
Flavobacterium.....	33	2
Micrococcus.....	36	7
Achromobacter.....	68	54
Pseudomonas.....	20	68
Proteus.....	0	9
Serratia.....	0	2

EXPERIMENT 3.

Forty-six cultures belonging to the genus *Flavobacterium* were isolated from frozen cod fillets. Forty-three of these did not reduce nitrite and three gave somewhat doubtful positive reactions.

EXPERIMENT 4.

Of sixty-two green fluorescent cultures isolated from ice, fresh water, sea water and fish, only nine were able to reduce nitrite.

EXPERIMENT 5.

Cod fillets containing 200 parts per million nitrite were left for 20 days in cold storage. By this time the fish had become spoiled and the nitrite entirely disappeared. Forty-six colonies were picked from plates made from this fish. Fifteen reduced nitrite and thirty-one did not.

EXPERIMENT 6.

It has been shown that the types of bacteria which predominate on fillets held in storage undergo a gradual change. On the fresh fillets the proportion of *Micrococci* and *Flavobacteria* is large, but after a few days in storage they rapidly become outnumbered by achromogenic gram-negative rods. In order to find out whether the nitrite-reducing capacity of the flora changed with the changing genera, tests were made on representative colonies picked from plates poured on successive days from untreated fillets stored at 3°C. The cultures isolated were from the plates of the highest dilution containing between 10 and 200 colonies. The results are shown in table I. During the first few days the nitrite reducers are relatively scarce, but at the point where the microflora changes the nitrite reducers become predominant.

TABLE I. The changes in the percentage of nitrite-reducing bacteria in colonies picked from high dilution plates poured on successive days from cod fillets stored at 3°C.

Days Stored at 3°C.	Number of Colonies Tested	Per Cent Positive
0	50	2
1	16	0
2	25	8
3	30	70
4	24	58.3
5	36	63.9
6	22	72.7
7	29	82.7
8	35	65.7
9	15	46.6
10	25	24.0
11	25	52.1
12	19	67.3

THE CORRELATION BETWEEN THE REDUCTION OF NITRITE AND THE REDUCTION OF TRIMETHYLAMINE OXIDE BY BACTERIA

Most of the bacteria which reduce the oxide also reduce nitrite; there are some species which reduce nitrite but not the oxide; and only a very small number reduce oxide, but do not reduce nitrite. As in all analyses of this type, the actual percentages varied from sample to sample, but the following observations are typical:

(1) Seventy cultures were isolated from cod fillets. These were tested with the following results:

67% reduced nitrite.

50% reduced trimethylamine oxide.

All those which reduced the oxide also reduced the nitrite; 17 per cent reduced nitrite but not oxide. Incidentally, all of these cultures which reduced nitrite but not oxide produced the green fluorescent pigment typical of the *Pseudomonas*.

(2) In a second series of tests, 316 cultures were isolated from cod fillets at intervals during a 12-day storage period at 0°C. Of these, approximately:

40% reduced nitrite.

15% reduced trimethylamine oxide.

This group of cultures differed from the previous series in that one-third of the oxide-reducing bacteria were unable to reduce nitrite. Fourteen out of nineteen of these oxide-reducing, but non-nitrite-reducing, bacteria were later identified as being Achromobacter. It was again observed that a very large percentage of those which reduced the nitrite but not the oxide were from green fluorescent colonies.

(3) With the stock cultures, identified to species, the results were approximately the same: All cultures of the *Enterobacteriaceae* that were examined reduced both the oxide and nitrite. The *Bacilli* reduced the nitrite but not the oxide. Most of those identified species of the *Achromobacteriaceae* and *Micrococcaceae* reduced neither oxide nor nitrite; but this was not always so with organisms isolated directly from the fish. At this point it might also be noted that of the cultures isolated from fish, almost all that could reduce nitrate also reduced nitrite; none was found that reduced nitrite but not nitrate. Most of the very few that reduced nitrate but not nitrite appeared to belong to either the *Micrococcaceae* or *Achromobacteriaceae* but never the *Enterobacteriaceae*.

The Influence of Nitrite-Reducing Bacteria or the Bacterial Reduction of Trimethylamine Oxide in the Presence of Nitrite

The effect of nitrite-reducing bacteria on the preservative action of nitrite can be clearly demonstrated by the use of pure cultures. In the following experiments this has been done in two ways: First, by using cultures that reduce either nitrite or the oxide, but not both, and comparing their oxide-reducing capacity separately and when combined; in the second series, organisms that can reduce both nitrite and the oxide are compared with others that reduce only the oxide.

(1) A nutrient broth containing 0.2% trimethylamine oxide and 209 p.p.m. sodium nitrite, having an initial pH of 6.9 was inoculated with two cultures (#29 and #P2) which reduced nitrite but not the oxide, one culture (#39) which reduced oxide but not the nitrite, and combinations of #39 with #29 and #P2. These were incubated at 3°C. and tested at 2, 6, 8 and 10 days for the production of trimethylamine (table II). By the eighth day the nitrite in cultures containing #P2 was almost entirely used up, while that in cultures containing #29 did not disappear entirely until after 12 days. It is quite evident from these, and from

TABLE II. The reduction of trimethylamine oxide in a nutrient broth having an initial nitrite content of 209 p.p.m., pH 6.9, inoculated singly and in combination with cultures that reduce the oxide or the nitrite, but not both.

Days at 3°C.	Trimethylamine Values				
	Cultures #29 (reduces Nitrite)	Cultures #P2 (reduces Nitrite)	Cultures #39 (reduces Oxide)	Cultures #29 + 39	Cultures #P2 + 39
2	0.44	0.51	0.51	0.50	0.46
6	0.49	0.65	2.36	3.00	6.14
8	0.48	0.69	4.4	14.8	10.6
10	0.49	0.73	17.7	17.7	29.0

other similar tests, that nitrite reducing bacteria have a very significant effect on the preservative action of nitrite, as far as the production of trimethylamine is concerned.

(2) The cultures capable of reducing trimethylamine oxide but not nitrite, are not only scarce, but of those that can be isolated from fish, many do not grow readily in the nutrient broths used in these experiments. For this reason special care was taken to obtain organisms of this type which did grow readily under these conditions.

TABLE III. The production of trimethylamine nitrogen, mg. per 100 ml. media, from trimethylamine oxide in the presence and absence of sodium nitrite (209 p.p.m.) by two cultures of bacteria that reduce only the oxide and six cultures that reduce both the oxide and the nitrite.

Days at 3°C.	Cultures reducing $(CH_3)_3$ NO and NO_2						Cultures reducing $(CH_3)_3$ NO but not NO_2	
	C_2	C_{22}	100	151	218	C_3	212	245
	Trimethylamine Values in Broth without Nitrite							
4	4.8	2.3	5.3	3.5	6.6	6.0	3.5	5.7
6	13.8	6.0	8.4	11.7	19.6	18.0	11.0	10.2
9	31.3	9.0	28.2	32.6	51.2	46.2	23.5	29.5
12	47.6	20.0	60.2	60.2	87.4	73.4	50.1	62.8
	Trimethylamine Values in Broth Containing Nitrite							
4	1.5	0.4	1.4	1.0	1.0	1.1	0.15	0.3
6	11.3	9.6	9.6	9.2	8.7	10.9	1.2	2.1
9	29.6	19.6	19.6	16.3	18.6	22.7	2.0	4.0
12	66.8	38.4	38.4	21.9	37.7	47.5	4.5	6.3

A nutrient broth, similar to Wood and Baird's (1943) oxide broth was made up having a pH 6.8 and 0.5% trimethylamine oxide. To one-half of this, sodium nitrite was added to give a NO_2 content of 209 p.p.m. and inoculated with two cultures which reduced oxide but not nitrite and six cultures which reduced both the oxide and the nitrite. These were incubated at 3°C. and tested periodically for trimethylamine. As shown in table III, the nitrite has a far greater retarding action on the production of trimethylamine where the cultures are unable to reduce the nitrite.

TABLE IV. The increase in trimethylamine in buffered trimethylamine oxide broths having the initial pH of 6.4, 6.8 and 7.2 to which 250 p.p.m. of $NaNO_2$ had been added, inoculated with an organism capable of reducing the oxide but not the nitrite, and stored at 0°C. for 28 days.

Days Storage	pH	TMA Value	Log of Bacterial Plate Count
0	6.4	0	5.64
	6.8	0	5.64
	7.2	0	5.64
5	6.5	.29	6.14
	6.9	.37	6.15
	7.2	.33	6.13
11	6.55	2.7	7.27
	7.0	3.0	7.64
	7.2	3.0	7.55
22	6.5	10.5	8.14
	7.0	12.5	8.21
	7.2	12.5	8.20
28	7.0	18.0	8.18
	7.4	17.2	8.25
	7.9	18.0	8.21

THE INFLUENCE OF PH ON THE NITRITE INHIBITION OF BACTERIAL REDUCTION OF TRIMETHYLAMINE OXIDE

Tarr (1942) has demonstrated that the bactericidal and bacteriostatic action of nitrite is dependent upon pH of the substrate. It is active in acid solutions, having little or no effect at a pH of 7.0 or above. This apparently does not apply to the inhibition of trimethylamine oxide reduction by bacteria as shown in table IV. Other tests using buffered oxide broth and initial pH's of 7.0 to 7.3 showed inhibition of oxide reduction in the presence of 150 to 200 p.p.m. sodium nitrite.

INFLUENCE OF THE SIZE OF INOCULUM

Interesting results were obtained by inoculating oxide broth (with and without 150 p.p.m. $NaNO_2$) with a series of dilutions made from a trimethylamine oxide-reducing culture isolated from cod muscle. The broth contained 0.5% oxide and was buffered at a pH of 7.3.

When the initial inoculum contained a large number of actively growing cells, the inhibition of oxide reduction by the nitrite was very slight. As the number of cells in the inoculum decreased, the inhibition became very pronounced (table V). After 24 hours' incubation, microscopical examination showed relatively little difference in the number of cells in each of the paired samples, with and without nitrite. They also showed that whereas a heavy initial inoculum quickly overcame the effect of the nitrite, the same or a greater number of cells resulting from the growth of an initially small number did not necessarily do so.

TABLE V. Effect of the size of inoculum on the reduction of trimethylamine oxide, in a nutrient broth with and without the addition of 150 p.p.m. sodium nitrite, and having an initial pH of 7.3.

Approximate Number of cells in the inoculum	Trimethylamine Values									
	24 hours		3 days		4 days		5 days		6 days	
	$-NO_2$	$+NO_2$	$-NO_2$	$+NO_2$	$-NO_2$	$+NO_2$	$-NO_2$	$+NO_2$	$-NO_2$	$+NO_2$
100,000	0.69	0.68	63.8	53.0						
10,000	0.27	0.19	35.4	30.7						
1,000	0.16	0.18	26.7	0.16	37.6	10.3	46.0	31.2	60.2	50.7
100	0.14	0.18	14.3	0.19	22.1	0.21	27.6	0.23	36.5	0.28
10	0.14	0.14	0.24	0.16	0.27	0.15	0.30	0.15	0.36	0.14

SUMMARY AND CONCLUSIONS

The normal microflora of cod fillets abound in nitrite-reducing bacteria. In freshly cut fillets they are less numerous, but during storage they soon constitute a large proportion of the total number of bacteria present.

Of the pure cultures tested, all the members of the family *Enterobacteriaceae* reduced nitrite. Of those identified as belonging to the genera *Achromobacter* and *Pseudomonas*, there were both nitrite-reducing and non-nitrite-reducing cultures. A very large majority of the cultures belonging to the *Micrococcus* and the *Flavobacterium* did not reduce nitrite.

Of the cultures tested, excluding the *Pseudomonas*, almost all that reduced trimethylamine oxide also reduced nitrite; a very few, in which the *Achromobacter* predominated, were able to reduce trimethylamine oxide but not nitrite.

By the use of pure cultures it was shown that nitrite-reducing bacteria interfere with the inhibiting action that small amounts of nitrite have on the

reduction of trimethylamine oxide by bacteria.

From these observations it is obvious that the value of nitrite as a preservative, especially in preventing trimethylamine production in fish, will vary with the activity and initial concentration of nitrite-reducing bacteria. As these organisms are normally present on fish and increase rapidly during storage, the sooner the nitrite treatment can be employed the more effective it will be. And, conversely, the use of nitrite dips for fish that is heavily contaminated, or almost at the spoilage stage, will be of little value.

At least for some species of bacteria, nitrite retards trimethylamine oxide reduction in pH ranges (6.9-7.1) where it is of little or no value as a bactericide or a bacteriostatic agent in the concentrations normally used.

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Further Studies on the Bacteriological Reduction of Nitrite in Fish During Spoilage

By W. J. Dyer and C. H. Castell
Atlantic Fisheries Experimental Station, Halifax, N.S.

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ABSTRACT

The reduction of added nitrite in stored cod fillets is due to bacterial agencies. There are two mechanisms of nitrite reduction, the first inhibiting bacterial growth in acid media, and the second inhibiting trimethylamine oxide reduction in neutral solution. The effect of several levels of nitrite concentration on spoilage in fillets stored at 3°C. as measured organoleptically and by spoilage tests was determined.

In a previous paper (Castell 1949) it was shown that concentrations up to 50 p.p.m. of sodium nitrite in cod fillets had no effect on the reduction of trimethylamine oxide to trimethylamine. Concentrations between 50 and 200 p.p.m. did inhibit the reduction of trimethylamine oxide but had no effect on bacterial growth. Much higher concentrations of nitrite were necessary to inhibit growth of the fish spoilage bacteria. Dyer (1949) showed that the nitrite was reduced in stored cod fillets treated with sodium nitrite and that rapid trimethylamine formation was inhibited until the nitrite concentration was reduced to the level of about 50 p.p.m. It was postulated that the effect of the nitrite was due to the action of nitrous acid or nitrite on the enzyme systems.

It is the intent of this paper to show that the reduction of nitrite is due to bacterial agencies and to present data on the relation between bacterial growth, reduction of nitrite and the production of spoilage products, such as trimethylamine, tyrosine, etc., in the range of nitrite concentration between zero and 200 p.p.m. in cod fillets.

In considering the reduction of nitrite, it should be pointed out that most studies on the effect of nitrite on the inhibition of spoilage have been carried out in fairly acid solution whereas in fresh cod fillets the pH varies between 6.2 and 7. (Dyer, Sigurdsson and Wood 1944). Since nitrous acid is a very weak acid, pK about 3.22 (calculated from data in Int. Crit. Tables p. 260), there is very little undissociated nitrous acid present at these pH values. From the above pK value it can be calculated that at a concentration of 200 p.p.m. sodium nitrite, about 2.2 p.p.m. nitrous acid is present at pH 5, about 0.22 p.p.m. at pH 6 and only 0.02 p.p.m. at pH 7. These values agree with those found by Philpot and Small (1938b) for higher concentrations of nitrite. Thus at pH levels of 6 or above there is very little nitrous acid present.

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Philpot and Small (1938) showed that tyrosine and phenol groups react with nitrous acid to form nitroso compounds which then react with nitric oxide, which is formed from nitrous acid, to yield diazo compounds. Most of their work was undertaken in acid solutions below pH 5, and with higher nitrite concentrations than we are dealing with in the present work, and it is more likely that reaction with amino groups would take place as has been discussed in a previous paper (Dyer 1949). However, this is a possible alternative for the formation of diazo groups as found by Sciarini and Nord (1945). Also Philpot and Small (1938b) have shown that reaction of the tyrosine groups in pepsin with nitrous acid does inhibit the peptic activity of the enzyme.

Thus a basis is provided for the well-known effect of nitrite in acid solutions in inhibiting bacterial growth, but Philpot and Small's work does not explain the effect of the nitrite in inhibiting the trimethylamine oxide reduction at a reaction near pH 7 where almost no nitrous acid is present. It seems probable that this is an entirely different problem which will be discussed later on in the paper.

EXPERIMENTAL

Is the Nitrite Reduction due to Fish Tissue or Bacteria?

In soil (Waksman, p. 456, 1932) and in sewage (Corbet and Wooldridge 1940) the reduction of nitrite has been shown to be almost entirely if not completely due to bacterial agencies.

To check whether there is any reduction of nitrite by the oxidation—reduction systems in the muscle tissues, a number of sterile samples of cod muscle were cut out and treated with sterile solutions of sodium nitrite and sodium nitrate to give concentrations of about 100 p.p.m. in the muscle tissue. It was found that on storage for 10 and 20 days at 5°C. there was no reduction of the nitrite or nitrate, while nonsterile control samples showed the usual reduction. Thus no appreciable reduction of nitrite by the enzyme systems present in muscle tissue was found. In addition the extent of reaction of the nitrite with amino or phenol groups was small enough not to be detectable by analysis.

It thus appears that the reduction of nitrite in stored fillets is due to bacterial agencies. There are large numbers of nitrite reducing bacteria in seawater (ZoBell 1932) and on fish (Castell 1949b). Of the spoilage bacteria in fish the *Pseudomonas* and *Achromobacter* were found to be strong nitrite reducers. Thus there is normally a flora in fish which is capable of reducing nitrite. Other data (Castell unpub.) show that the higher the count the greater is the rate of reduction of the nitrite concentration.

Reduction of Nitrite and Spoilage Changes

Fresh cod fillets from a local fish company were dipped one minute in cold solutions of sodium nitrite to give a range of nitrite concentrations in the fillets in the order of 0 to 200 p.p.m. The following concentrations of sodium nitrite were used; A—0 (control) B—0.026%, C—0.07%, D—0.12%, E—0.21%. The fillets were drained, wrapped in waxed paper and stored at 3°C. Samples were taken every 1 or 2 days. Composite samples were obtained by cutting equal

portions from each of 3 fillets in each treatment. For total bacterial counts 50 g. of the composite sample was blended in the Waring Blendor with 50 ml. sterile water, and aliquots pipetted off. The numbers of trimethylamine oxide reducing bacteria were estimated by inoculating Wood and Baird's oxide broth with serial dilutions of the above blended sample, and after suitable incubation, recording the dilution showing positive trimethylamine oxide reduction (Wood and Baird, 1943). Solutions were made for nitrite determination (Dyer 1946) by adding water to 40 g. of the blend to make a total volume of 500 ml. and blending with the Waring Blendor. An additional 50 g. aliquot of the blend was mixed with 50 ml. 10% trichloracetic acid for estimation of trimethylamine (Dyer 1945) and of tyrosine (Wood, Sigurdsson and Dyer 1942). The presence or absence of nitrate was checked by reducing with zinc dust followed by addition of the nitrite reagents (ZoBell 1932). Organoleptic examination of the samples was also made.

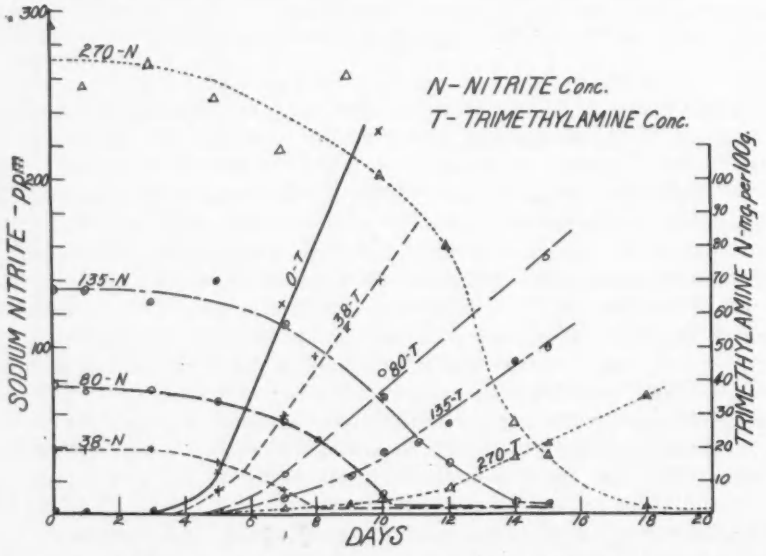

FIGURE 1. Nitrite reduction and trimethylamine increase in cod fillets of initial sodium nitrite content of 0, 38, 80, 135 and 270 ppm stored at 3°C. N—nitrite concentration. T—trimethylamine concentration. X—initial nitrite content O. +—initial nitrite content 38 ppm. O—initial nitrite content 80 ppm. Θ—initial nitrite content 135 ppm. Δ—initial nitrite content 270 ppm.

In fig. 1 is shown the relation between nitrite reduction and trimethylamine increase during storage. The initial nitrite concentrations were 0, 38, 80, 135, and 270 p.p.m. in the five treatments. It is evident that the presence of the nitrite delays the reduction of trimethylamine oxide confirming the results

previously obtained in broth cultures (Castell 1949). Even at as low a concentration as 38 p.p.m. sodium nitrite, there is a definite retardation of the oxide reduction, about one half day over the control, whereas at 270 p.p.m. initial nitrite concentration the delay of rapid trimethylamine formation is about 9 days over the control. A comparison of the time required for the reduction of nitrite to 50 p.p.m., rise of trimethylamine to 15 mg. N per 100 g., rise of pH to 7, increase of tyrosine equivalent to 16 mg. tyrosine per 100 g. fish, and the organoleptic borderline between acceptable and spoiled, is shown in table I.

TABLE I. Time required, days storage at 3°C. for change.

Initial nitrite conc.	Nitrite reduced to 50 p.p.m.	TMA rise to 15	pH rise to 7.0	Tyrosine rise to 16	Organoleptic border line
0	—	5.2	5	5.5	5-5.5
38	—	6	6	6	7-7.5
80	7.5	7.3	6.5	7.5	8-8.5
135	11	9.5	8	9.5	10-11
270	14	14	15	11	12-14

It will be observed that the effect on the keeping quality is proportional to the initial nitrite concentration. The increased keeping time as estimated organoleptically agrees very well with the biochemical evidences of spoilage changes. In addition to delaying the formation of trimethylamine, the nitrite also delays the rise in pH, which of course is largely dependent on the trimethylamine oxide reduction (Dyer, Dyer and Snow 1946) and also the rise of the tyrosine equivalent which has been used as a rough measure of the degree of proteolysis in fish by Bradley and Bailey (1940) and by Wood, Sigurdsson and Dyer (1942). It should be pointed out that the tyrosine reagent reacts with many reducing substances such as tyrosine, purines, hydrogen sulfide, trimethylamine, etc., and while the rise in tyrosine value is usually parallel to proteolysis in fish tissue, the actual reducing action is certainly not all due to tyrosine. It is therefore used as an approximate indication of the degree of proteolysis.

At all levels of nitrite content, the nitrite is reduced during storage slowly at first and then more rapidly. It will be seen from fig. 1, that the nitrite has been reduced to a concentration of about 50-100 p.p.m. before the stage of rapid trimethylamine increase occurs, in agreement with our previous results (Castell 1949, Dyer 1949).

In fig. 2 is shown the rise in tyrosine values. These values are affected by the nitrite in much the same way as the trimethylamine formation. However it will be noted that at the higher nitrite concentrations the inhibition of tyrosine formation is not so great as the effect on the trimethylamine oxide. This is shown in fig. 3 where the ratio between the tyrosine and trimethylamine formation is

shown. In fig. 3 the tyrosine values have been corrected for the contribution made by the trimethylamine itself. While the nitrite does definitely delay the rise in tyrosine value, the effect is much less than the inhibition of trimethylamine

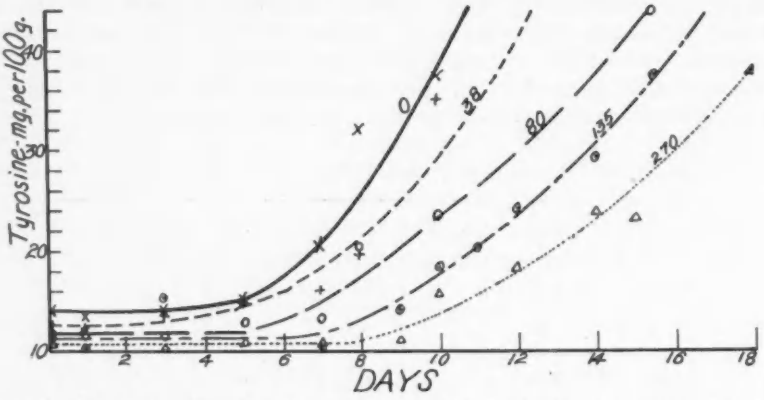

FIGURE 2. Rise in tyrosine values in cod fillets containing 0, 38, 80, 135 and 270 ppm sodium stored at 3°C.

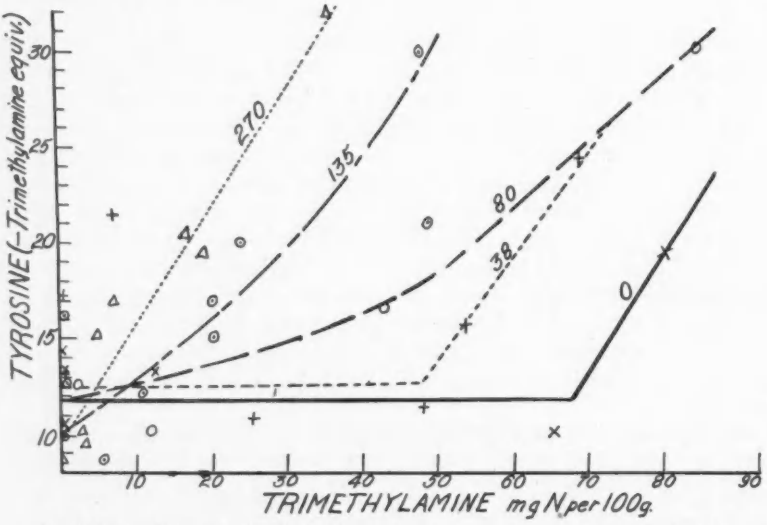

FIGURE 3. Relation between trimethylamine concentration and tyrosine equivalent (corrected for trimethylamine) in cod fillets containing 0, 38, 80, 135 and 270 ppm sodium nitrite stored at 3°C.

oxide reduction. In the control fish as is usual (Dyer and Mounsey 1945), no appreciable proteolysis occurs until the trimethylamine has reached a level of about 60 mg. N per 100 g. However in the presence of the nitrite this level is

progressively lowered. At 38 p.p.m. initial nitrite concentration, rapid tyrosine formation begins at a level of about 50 mg. trimethylamine nitrogen per 100 g. At 80 p.p.m. nitrite, the trimethylamine level is about 10, and at 135 and 270

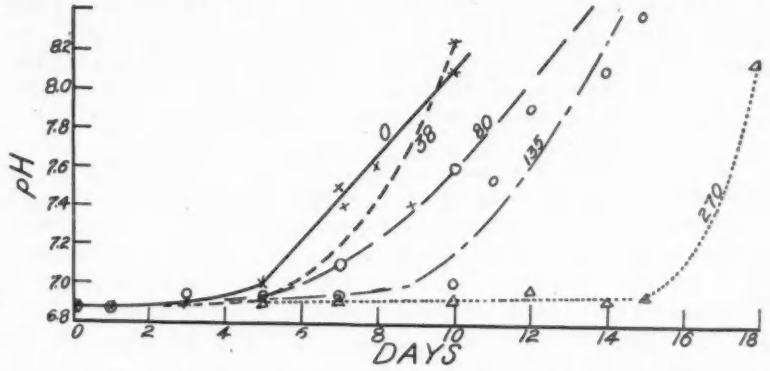

FIGURE 4. Rise in surface pH values in cod fillets containing 0, 38, 135 and 270 ppm sodium nitrite stored at 3°C.

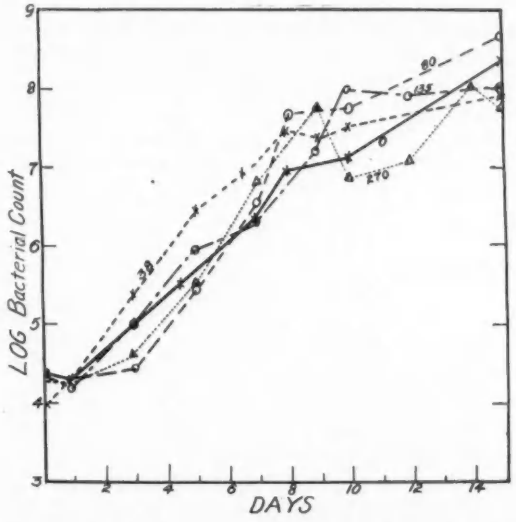

FIGURE 5. Bacterial count in cod fillets containing 0, 38, 80, 135 and 270 ppm sodium nitrite stored at 3°C.

p.p.m. nitrite both tyrosine and trimethylamine formation begin at the same time. Thus in nitrite-treated fish stored at about the limit of its keeping time the tyrosine value is apt to be much higher than in untreated fish stored to about the same spoilage level as indicated by the concentration of trimethylamine. This

is adequately supported by other observations made in this laboratory. It also emphasises the fact that the interpretation of spoilage tests must be made with due recognition of all the factors involved in any particular case.

The changes in pH are shown in fig. 4. These parallel the changes in trimethylamine and in tyrosine both of which directly affect the pH. The formation of the alkaline trimethylamine base, the removal of the strong buffer the trimethylamine oxide, raises the pH, as also does the formation of ammonia during proteolysis. As soon as the buffering power of the fish muscle is overcome there is a rapid rise in pH.

The changes in bacterial count are shown in fig. 5. The total bacterial count remains almost stationary for the first day, then increases rapidly for about 8 or 9 days, then slows down but still increases to about 15 days, at which time the count has reached about 100,000,000 bacteria per g. There seems to be no significant difference in bacterial count or growth rate with the different nitrite concentrations. The conclusion is that there is little or no inhibition of bacterial growth by nitrite in the concentrations used. This is an agreement with former data (Castell 1949).

In order to show that there was no selective effect on the bacteria resulting in the elimination of trimethylamine oxide reducing bacteria, which might explain the inhibition of trimethylamine formation by the nitrite treatment, the number of oxide reducing bacteria were determined by making broth dilution counts and noting the highest dilution showing trimethylamine oxide reduction. This gives an approximation of the number of bacteria present capable of reducing the oxide. The results are shown in table II.

TABLE II. The numbers of oxide-reducing bacteria compared with the total numbers of bacteria in the fish at successive storage periods as shown by "dilution counts" in trimethylamine oxide broth.

Days	Initial nitrite concentration, p.p.m.									
	0		25		50		100		200	
	A	B	A	B	A	B	A	B	A	B
0	5	3	5	4	5	3	5	4	4	2
1	5	4	5	5	5	3	5	5	5	3
3	6	6	6	5	6	5	6	6	6	5
7	7	7	8	7	8	7	7	7	8	6
10	8	8	9	9	8	8	9	9	8	8
15	9	8	9	8	9	9	8	8	8	8

A—Highest dilution showing growth.
B—Highest dilution showing oxide reduction.
These values are the negative logarithms of the dilution.

It will be seen that the oxide reducing bacteria make up about one tenth of the total bacteria at the beginning, and the proportion increases slightly on storage. The inhibition of trimethylamine oxide reduction in the presence of

nitrite was thus not due to a suppression of the bacteria which are capable of reducing the oxide.

From fig. 1 it is evident that the rate of trimethylamine formation is much slower in the nitrite treated samples than in the control samples. There does not seem to be any reason for this effect, since we have shown elsewhere (Dyer 1948) that the number of oxide reducing bacteria is greater rather than less as spoilage proceeds. We do know that there is a rapid change in the bacterial flora in stored fillets, and it may be that this influences the rate of trimethylamine formation even though the proportion of oxide reducers is not lowered.

There is adequate evidence for the effect of nitrous acid in inhibiting bacterial growth in acid medium (Meiklejohn 1940, Tarr 1941 etc.) but in the present case we are dealing with media close to neutrality, where there is little interference with bacterial growth in nitrite concentrations below 200 p.p.m., but there is a significant inhibition of trimethylamine oxide reduction. Under these conditions we have shown that there is almost no nitrous acid present and this inhibition must be due to the nitrite itself. It seems that the mechanisms involved in the two cases are different. We have shown (Dyer 1949) and in the present paper that the trimethylamine oxide is not reduced until the nitrite has almost disappeared. This effect may be associated with the relative poising action of the trimethylamine oxide-trimethylamine oxidation-reduction system and the nitrite-hydroxylamine system, but in such a heterogeneous media where the potentials at sites on the bacterial cell control the reactions it is difficult to make a theoretical interpretation, especially since the oxidation-reduction systems involved may not be truly reversible.

SUMMARY

The reduction of nitrite in stored cod fillets was found to be due to bacterial agencies and no chemical reduction could be detected.

In fillets containing 0, 38, 80, 135 and 270 p.p.m. sodium nitrite stored at 3°C., the nitrite was reduced to 0 in from 8 to 18 days, the higher the nitrite concentration the longer the time taken for reduction. The formation of trimethylamine was inhibited until the nitrite concentration was reduced to about 50 p.p.m. The tyrosine and pH changes were similarly affected by the nitrite although tyrosine formation was not inhibited to the same extent as the trimethylamine. The total bacterial count was almost unaffected by the presence of the nitrite, the count in treated samples increasing at the same rate as the control. In addition, the bacteria reducing trimethylamine oxide were not inhibited by the nitrite, so that the nitrite directly affects the enzymic reduction of the trimethylamine oxide. It is concluded that two mechanisms are concerned. 1. The inhibition of the growth of bacteria in acid solutions where nitrous acid is present, and 2, the inhibition of the reduction of trimethylamine oxide near neutrality where almost no nitrous acid is present.

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Effects of Water Circulation on the Growth of Quahaugs and Oysters

By C. J. Kerswill
University of Western Ontario

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ABSTRACT

Holding quahaugs and oysters through a growing season in circulation-controlled trays in Bideford river, Prince Edward Island, showed that they react differently to decreased water circulation. Quahaugs grow in proportion to the extent of circulation and can survive and grow significantly when there is very little water flow. Oysters are affected little by moderately reduced circulation but grow little and may die when the circulation is much reduced. Growth of quahaugs in beds is seriously reduced by eel-grass growing on the beds, and also by eel-grass merely in the vicinity of the beds. Growth curves are given for quahaugs in natural up-river and down-river beds and the difference in growth rate is attributed to difference in water circulation.

Several investigators have reported that the growth rate of bivalve molluscs is more rapid where there is active water circulation over the beds than where the circulation is sluggish. Examples are the observations of Belding (1912) on the growth of quahaugs (*Venus mercenaria* L.) in Massachusetts, and those of Fraser and Smith (1928) on butter clams (*Saxidomus giganteus*) and little neck clams (*Paphia staminea*) in British Columbia. There are few published records of data obtained in experiments designed to isolate the effect of water circulation as a factor limiting growth rate. An opportunity to conduct such experiments was afforded the author in 1939 and 1940 during the course of a general investigation of the environmental factors limiting the growth and distribution of the quahaug in eastern Canada. Headquarters for the field work was the Prince Edward Island Biological Station, Ellerslie, P.E.I., then under the direction of Dr. A. W. H. Needler, whose assistance is gratefully acknowledged. In the winters the results were analysed during the course of graduate studies at the Department of Zoology, University of Toronto, where valuable assistance was received from Dr. A. G. Huntsman and other staff members.

Early in the investigation observations on the growth rate of specimens obtained from natural beds in various areas and of marked specimens planted in experimental plots and trays indicated the importance of water circulation as a factor limiting growth (Kerswill, 1942). Areas suitable for such observations occurred in Bideford river, an inlet of Malpeque bay. Here, limited quantities of quahaugs could be fished in the upper branches, such as Paugh's creek, where water circulation was poor owing to limited tidal exchange and the presence of dense eel-grass, and also in down-river areas where there were considerable tidal currents and eel-grass was practically absent. In 1940 an experiment was designed, using special boxes set out on an intermediate area in the river, to show the

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effect of different degrees of water circulation on the growth of quahaugs, and for comparison, samples of oysters (*Ostrea virginica* L.) were included. The data of this and other growth experiments are presented, followed by a discussion of the growth rate of quahaugs on natural beds in relation to water circulation.

EXPERIMENTAL METHODS

The method generally used in the quahaug growth experiments consisted of holding quite large samples, selected to have initially similar size-frequency distributions, under certain conditions throughout the growing season. At the end of the season the mean sizes of the various lots were compared. As an index of size, the height of the shell (the greatest distance from the umbo to the ventral edge of the valves as determined by vernier calipers) was used throughout rather than length. This was done because it was sometimes desired to measure to the edges of successive growth rings on a particular specimen to determine the size reached in different years. For this purpose in quahaugs, less error is involved in the height measurement than in the measurement of length.

The standard errors of the mean heights were calculated to show the variability of each sample and for use, when desired, in calculating the significance of differences between means. All the specimens were obtained in Paugh's creek within a radius of about 200 yd. (1 yd. = 0.91 m.), and their average age, determined from growth rings, was about six years at the start of the experiments. The experiments were set up in Bideford river within a distance of 500 yd. from the biological station, and about 1000 yd. from where the specimens had been obtained.

Plots and Trays

Rectangular plots, 24 in. by 48 in. (1 in. = 2.5 cm.), with the corners marked by stakes, were planted in various places below low tide level. Wooden trays of the same dimensions with sides six inches high, and painted to resist shipworms, were filled with sandy mud and either placed on the bottom or suspended from tripods at various depths. When such a tray on the bottom was planted with specimens, the latter were 6 in. higher than specimens in adjacent plots. In 1939 the number of specimens planted in each plot or tray was 50, but in 1940 the number was 73. All were notched lightly on the ventral margins of the valves according to a code that served to identify each lot.

Special Boxes Used in 1940

Three covered boxes, similar except for the ends, were made of one-half inch lumber with dimensions 44 in. long, by 24 in. wide, by 12 in. high. The ends of the first were covered with fox wire of one-inch mesh; the ends of the second with wooden slats one inch wide and spaced one-half inch apart, and the ends of the third were boarded over and six one-half inch holes were bored, evenly spaced, in each end. After painting for protection, a layer of concrete one inch thick was placed in the bottom as ballast. Inner trays of one-half inch

galvanized wire screening, with dimensions 24 in. long by 18 in. wide by 4 in. high, and divided longitudinally by screening into two similar compartments, were suspended firmly by wires inside the boxes to give a uniform space between tray and box all around. Tarred rope bridles were attached for manipulation of the boxes, and white stripes were painted on the covers to check their alignment when placed on the bottom at a depth of about three feet of water at low tide.

At the start of the experiment similar lots of 73 quahaugs belonging to the series used in the plots, were placed on the wire of one compartment of each tray; in the other compartments were placed similar lots of 75 measured oysters (1939 spat). The oysters were measured in two dimensions, length and width, which were averaged because of the natural irregularity of the shells. The boxes were lowered onto an oyster bed just east of the biological station where there was no eel-grass or other obstruction for at least 35 yd. in any direction, and arranged parallel to one another 6 in. apart, with the long axes parallel to the direction of the prevailing tidal currents. The alignment was checked regularly and was found to remain constant throughout the summer. No fouling by sea weeds or other materials occurred.

RESULTS OF EXPERIMENTS

Table I shows the data of all the experiments conducted in 1939 and 1940 which are considered to have a bearing on water circulation. The order of the plots or trays in each series has been arranged arbitrarily to begin with the fastest growing lot of specimens, followed by the other lots in descending order as regards growth.

Growth of Quahaugs

Most of the differences in growth rate among the various lots of quahaugs can be interpreted by considering that water circulation is the principal factor involved. The significant features of the data are as follows:

Specimens planted in trays placed on the bottom grew appreciably more than those set in plots, other conditions being the same. The tray specimens were 6 in. higher than those living in the bottom and would be subjected to stronger currents. An indication of the increase in current with increased distance from the bottom was obtained from current readings with a Gurley meter, which are discussed more fully later.

Specimens planted in plots among heavy growths of eel-grass grew very little as compared to specimens planted in clear bottom; plots in light eel-grass grew an intermediate amount, and a plot planted in 1940 on clear bottom, but near a heavy growth of eel-grass, was affected similarly to the plot in light eel-grass. It is considered that the eel-grass seriously impeded water circulation over the specimens, causing a decrease in their food supply. Similar observations were made by Belding (1912).

Specimens planted in suspended trays arranged in a vertical series grew more rapidly near the surface of the water than at or near the bottom. Growth in the

TABLE I. Results of 1939 and 1940 growth experiments.

Year, and Specimens	Position	Mean height at start of expt. ± standard error (mm.)	Mean height at end of expt. ± standard error (mm.)	Growth (mm.)
1939, Quahaugs		(June 1st)	(Sept. 15th)	
	Tray on clear area	28.6 ± 0.6	39.5 ± 0.6	10.9
	Plot in clear area	28.8 0.7	36.8 0.5	8.0
	Plot in light eel-grass	28.2 0.6	34.4 0.6	6.2
	Plot in heavy eel-grass	32.0 0.4	35.2 0.4	3.2
1940, Quahaugs		(June 11th)	(Sept. 12th)	
	A. Plots and trays on bottom just below low tide level.			
	1. Tray on clear area	31.3 ± 0.6	39.7 ± 0.6	8.4
	2. Plot on clear area	31.3 0.8	38.1 0.7	6.8
	3. Tray in light eel-grass	31.0 0.8	36.8 0.6	5.8
	4. Plot near heavy eel-grass	31.0 0.7	36.5 0.6	5.5
	5. Plot in heavy eel-grass	31.8 0.6	33.1 0.6	1.3
	B. Trays in vertical series, middle of river, depth of water 10 feet at low tide.			
	1. 7½ feet above bottom	31.4 ± 0.6	40.4 ± 0.5	9.0
	2. 5 feet above bottom	31.0 0.7	39.6 0.6	8.6
	3. 2½ feet above bottom	31.8 0.7	39.2 0.6	7.4
	4. On bottom, clear area	30.8 0.9	38.1 0.6	7.3
	C. Wire-bottom tray floating at middle of river	30.9 ± 0.6	35.0 ± 0.6	4.1
	D. Special boxes on bottom, depth of water 3 feet at low tide.			
	1. Box with open ends (wire)	31.1 ± 0.8	35.7 ± 0.6	4.6
	2. Box with slat ends	30.8 0.8	34.3 0.5	3.5
	3. Box with solid ends, except for small holes	31.6 0.7	33.0 0.6	1.4
1940, Oysters		Mean (L+W)/2 (June 11th)	Mean (L+W)/2 (Sept. 12th)	
	E. Same as D above, in parallel compartments.			
	1. Box with open ends (wire)	24.7 ± 0.3	48.9 ± 0.7	24.2
	2. Box with slat ends	24.7 0.3	47.6 0.7	22.9
	3. Box with solid ends	24.7 0.3	28.1 0.6 (34 dead E3)	3.4

tray at mid-depth, 5 feet above bottom, (B 2) was of the same order as growth in the tray on clear bottom just beyond low-tide level (A 1); growth in the highest tray (B 1) was the fastest of any lot in the 1940 experiments, likely because of good water circulation and good provision of food. These results should be compared with those obtained for quahaugs placed in the wire-bottom tray (C) floating at the surface, in which the specimens merely lay on their sides on wire screening about 6 in. under the water surface. This tray was provided with a lid, and was similar to those used successfully for the rapid rearing of oyster spat in similar places. Quahaugs do poorly when held in this manner, likely because their intake of water through the siphons is hindered when the animal is not in its normal upright position.

In the experiment where the special boxes were used to control the amount of water circulation, the importance of the latter as a factor affecting growth rate is clearly shown. Maximum growth occurred in box D 1 where water circulation was practically unimpeded, and minimum growth in D 3 which had almost no water circulation; the former grew similarly to lot C in the floating tray, as would be expected, and the latter grew very little, like the plot in heavy eel-grass (A 5). Considering the growth in box D 1 as 100%, the growth of quahaugs in boxes D 2 and D 3 was 76% and 31% respectively.

It is of importance to note that there was no mortality among the quahaugs even in the closed box (D 3), or when they were held among heavy eel-grass.

Growth of Oysters

The results of the experiment where special boxes were used (E) show that oysters and quahaugs react differently to variations in water circulation. In the wire-end box, D 1, the oyster spat almost doubled their size in the three-month period, and those in E 2 grew as much to within 5%. Thus, oyster spat are not affected by a moderate decrease in water circulation to as great an extent as are quahaugs; in the latter a difference of 24% occurred in the growth of lots D 1 and D 2. Oyster spat in the closed box, E 3, were severely affected by the reduction in water circulation. Only 41 of the 75 specimens survived the summer, and the survivors grew only 14% as much as those in box E 1.

GROWTH OF QUAHAUGS IN NATURAL BEDS

A consideration of data on the growth rates of quahaugs living under natural conditions is necessary for an understanding of the significance of the growth experiments. Such data can be obtained readily for Canadian quahaugs since the growth-ring method of age determination can be used successfully. It is necessary only to collect a sample of about 100 specimens at one place and to measure on each, with vernier calipers, the size of the shell to the end of each year of growth. The measurements are then grouped into year classes and plotted as age-size curves.

This was done for samples taken in May, 1939, from (*a*) a bed in Paugh's creek, the up-river inlet of Bideford river already mentioned, and (*b*) a bed $3\frac{1}{4}$

miles down-river (1 mile = 1.61 km.) near Port Hill wharf. The data are shown in fig. 1, where height-frequency polygons have been plotted along vertical axes to show the variation in size at the end of each year's growth, and growth curves

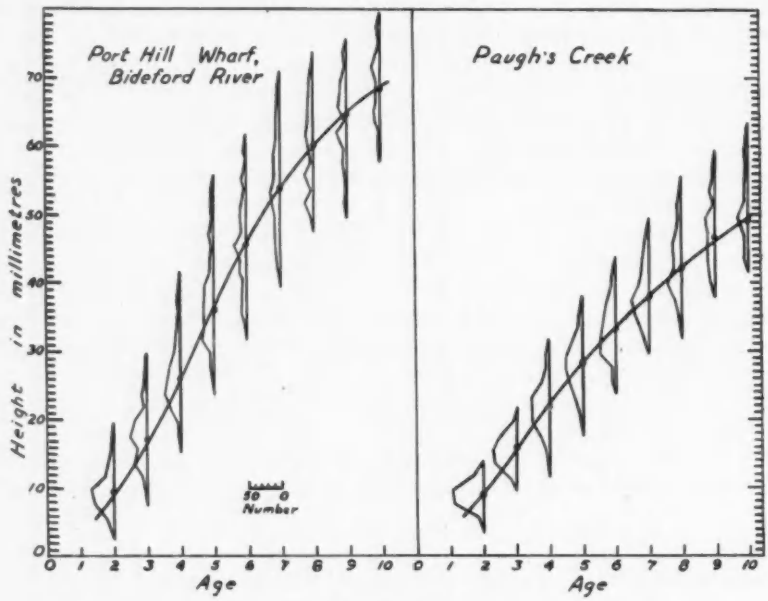

FIGURE 1.

have been drawn through the mean heights attained each year. A marked difference is seen between the growth rates at the two places, showing that Bideford river near Port Hill wharf had much more favourable growing conditions than had Paugh's creek.

DISCUSSION

It appears that the difference in the growth rates of quahaugs living in Bideford river in up-river and down-river beds can be attributed to differences in water circulation, which are quite marked. At Port Hill wharf, the river is about three-quarters of a mile wide, and through it strong tidal currents run between Malpeque bay and the upper tributary creeks. In 1940 it was found that currents here were sufficient to give significant readings with a Gurley current meter, the vane of which will not revolve uniformly at velocities under 0.25 ft. per second (1 ft. = 3 dm.). For example, on August 23, at rising half tide, a velocity of 0.66 ft. per sec. was read at a distance of 6 ft. above bottom and at greater distances above bottom no appreciable differences, attributable to current only, could be found. At lower levels the velocity decreased regularly, for example, at distances above bottom of 4 ft., 2 ft., and 2 in. respectively, the velocity was 0.59, 0.43, and 0.25 ft. per sec. This would explain on the basis of

water circulation, the difference in growth rate mentioned previously between quahaugs held a few inches above bottom and those planted in the bottom. In Paugh's creek tidal currents over the quahaug beds are almost negligible since the head of tide is only three-quarters of a mile up-stream, and the width of the inlet is under 300 yd. Here the vane of the Gurley meter would not even turn at any stage of tide, whereas to give the value of 0.66 ft. per sec. at Port Hill wharf it turned at the average rate of 10 revolutions per 37 sec.

Other factors such as temperature and salinity were considered as possibly influencing the growth rate of quahaugs at down-river and up-river positions, but it seems unlikely that they would have significant effects in this case (Kerswill, 1942). During the short growing season for quahaugs in Canada, about June 1st to September 15th, very little difference between either temperatures or salinities has been observed at the two places. Usually the temperature in Paugh's creek is slightly higher than at Port Hill wharf but the difference seldom exceeds 2°C. The salinity in Paugh's creek is generally slightly lower than that down-river, but during the summer the difference seldom exceeds two parts per mille and that difference occurs only for short periods immediately after heavy rains. Further evidence for the similarity of water temperatures and salinities over up-river and down-river areas in Bideford river is given by Medcof (1946).

The quahaugs in Paugh's creek were not inherently slow-growing, because their growth rate greatly increased after they were transferred to the plots near the biological station for the experiments described above. Fig. 1 shows that Port Hill wharf quahaugs grew about 10 mm. in height between the fourth and fifth years of age. Height increases of this order, namely 8.4 mm. to 9.0 mm., occurred in the case of Paugh's creek quahaugs of about the same size and five to seven years old, with an initial average height of 31 to 32 mm., when transferred to trays near the biological station for the 1940 growing season (table I—A 1, B 1, B 2). If left in Paugh's creek their expected mean height increase during 1940 would be only about 5 mm. Thus, growing conditions which led to the best results in the experimental plots and trays almost equalled the favourable natural conditions at Port Hill wharf. At the other extreme was the poor growth of quahaugs at the same experimental site but subjected to little water circulation. One can only speculate on the reason for the important effect of water circulation on the growth of quahaugs but it is doubtless related to the availability of food, and perhaps, under some circumstances, to the cleansing of the exposed ends of the siphons.

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Bacteriological Peptones from Fish Flesh

By H. L. A. Tarr and Catherine P. Deas
Pacific Fisheries Experimental Station
Vancouver, B.C.

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ABSTRACT

Peptones were prepared from fish flesh by hydrolysis with (1) tryptic or peptic enzymes derived from fish digestive tracts, (2) inorganic acids followed by neutralization of the reaction mixture with an ion exchange resin, calcium hydroxide or barium hydroxide, and (3) sodium hydroxide followed by neutralization with an ion exchange resin. It was found that only those peptones which were prepared by enzyme hydrolysis consistently supported good growth of six *Streptococcus haemolyticus* cultures and of one *Clostridium botulinum* type E culture, and that these peptones were generally superior to three commercial peptones in supporting growth of the haemolytic streptococci.

Numerous bacteriological peptones are marketed, but technical information concerning the materials and methods used in their preparation is not usually divulged by the manufacturers. One firm has published fairly detailed analyses of their peptones (Difco 1943) but it is by no means clear whether there is any relation between these analyses and the ability of the peptones to support bacterial growth. Bacteriological peptones normally contain extremely variable amounts of the ill-defined products of protein hydrolysis (proteoses, peptones and polypeptides, as well as amino acids and ammonia), soluble tissue extractives, B vitamins, inorganic salts and, possibly, carbohydrates in small amounts. In the light of the newer knowledge of bacterial nutrition it would appear reasonable to assume that the ability of a given peptone to promote bacterial growth would depend more upon its content of essential amino acids, B vitamins and other growth factors than on the degree of hydrolysis of the protein used in its preparation.

Hook and Fabian (1943) have reviewed the literature pertaining to bacteriological peptones thoroughly, and it appears that fish flesh has rarely been used in their preparation. Vande Velde (1940) obtained a fish peptone by neutralizing a hydrochloric acid hydrolysate of fish flesh with sodium hydroxide and drying the solution to a powder. Fish muscle tissue is known to be a valuable source of essential amino acids (Deas and Tarr 1947 and 1948, and submitted for publication 1949) and in the form of a tryptic digest formed an excellent medium for the cultivation of certain strict and facultative anaerobic bacteria (Tarr 1942). For these reasons the possible value of fish flesh in preparing bacteriological peptones was investigated. Reference to this work has already been made (Tarr 1948, Tarr and Deas 1948).

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EXPERIMENTAL

Skinned fillets of different species of fish were employed as starting material and, in some cases, these were leached in running tap water (5 to 8°C.) prior to use. It was thought that leaching might exert a favourable effect in that it would tend to remove any soluble reducing carbohydrates which, if present, might interfere should the peptones be required for fermentation tests. This procedure would probably also counteract any tendency for a Maillard (amino-aldehyde) type of reaction to occur during preparation, and thus to avoid the formation of dark-coloured peptones. On the other hand it was realized that leaching could prove undesirable in that a loss of water-soluble B vitamins or other growth factors required by certain bacteria might result. Also this procedure would undoubtedly occasion some loss of water-soluble muscle myogen from ruptured muscle cells.

The flesh was prepared for hydrolysis by blending it in a Waring Blendor, the required amount of water, acid or alkali being added at this stage. Where enzyme hydrolysis was employed, the watery flesh suspension was heated to 100°C. for 5 to 10 minutes to destroy microorganisms which might occasion putrefaction during incubation if they were imbedded in the flesh particles, and also to denature the flesh proteins and thus render them more susceptible to enzyme attack. Since fish stomach tissues contain pepsin (Norris and Elam 1940), and their pyloric caeca contain tryptic enzymes (Johnston 1941), these convenient sources of proteolytic enzymes were employed. The respective organs were removed from strictly fresh fish, lightly washed with cold water, and then blended finely for direct addition to the heated flesh preparations. It was felt that no useful object could be attained by partially purifying or concentrating the enzymes for preparation of peptones. After the addition of the required quantity of enzyme preparation and a layer of toluene as bacteriostat, the flesh suspensions were incubated at 38°C. Vigorous and fairly frequent shaking was employed, especially during the first days' incubation in order to retard or prevent bacterial multiplication. The details of preparation of the individual peptones vary considerably so these have been tabulated separately below. Drying of the hydrolysed solutions was accomplished as follows. The clarified, neutralized filtrates (pH 7.0 to 7.2) together with the washings obtained from undigested material or from the ion exchange resins, were concentrated by evaporating to a light syrup in a porcelain dish exposed to an air current, the temperature of the liquids not being permitted to rise above 100°C. The syrups were stored for one day at 0°C. and were then filtered with suction through a thin layer of "Filtercel" in order to remove insoluble material. The insoluble residues which collected on the filters were washed with a little cold water, the washings being added to the filtrates. The insoluble materials, except in the case of peptone 1 (see enumerated list below) were discarded. The clear filtrates were washed into tared evaporating dishes, and these were dried in vacuo, first over H_2SO_4 and finally for at least two weeks over P_2O_5. Vacuum spray or tray drying would undoubtedly prove more suitable for large operations. The resulting peptones were finely pulverized

in a mortar and, since all, especially those prepared by enzyme hydrolysis, were hygroscopic, they were stored over P_2O_5 in vacuo. The yields were calculated from the dry weight of the flesh, or flesh plus enzyme preparation. The details of preparation of the individual peptones are tabulated as follows.

Peptone No.	Fish used	Method of preparation	Yield %
1	Lingcod (*Ophiodon elongatus*)	Flesh leached 2 days (500 g.), H_2O (500 ml.), lingcod caeca (10 g.) and 5N NaOH (20 ml.) digested 17 days. Initial pH 9.5, final pH 7.4.	54
2	"	Undigested residue from preparation of peptone 1 (77 g. wet weight) and 400 ml. 1N HCl hydrolyzed 5 hr. at 120°C. Adjusted pH of filtrate to 6.5 with 180 g. Amberlite anion-exchange resin IR4B adjusted to the sodium cycle.	35 (calculated on starting material for peptone 1)
3	"	The material used and procedure followed were the same as for peptone 1, except that 20 g. of lingcod caeca were added and the digestion period was 3 days.	39
4	"	Flesh leached 2 days (500 g.) and 2N H_2SO_4 (500 ml.) hydrolyzed 3 hrs. at 120°C. The sulphate in the filtrate was removed quantitatively with baryta and the precipitates washed.	39
5	Chum salmon (*Oncorhynchus kela*)	Flesh leached 2 days (1500 g.), H_2O (1500 ml.), chum salmon caeca (60 g.) and 5N NaOH (35 ml.) were digested 1 day and one half the suspension was processed. Initial pH 9.5.	47
6	"	The remainder of the suspension used to make peptone 5 was digested for an additional 12 days before processing.	69
7	"	Flesh leached 2 days (500 g.), H_2O (500 ml.), chum salmon stomachs (100 g.) and 5N HC1 (20 ml.) digested 12 days. Initial pH 2.0.	59
8	Starry flounder (*Platichtys stellatus*)	Flesh (400 g.), H_2O (400 ml.) chum salmon caeca (20 g.) and 5N NaOH (16 ml. added in three separate portions during the first 3 days' digestion) were digested for 11 days. Initial pH 9.0, final pH 8.0.	70
9	"	Flesh leached 1 day was digested under conditions similar to those followed in preparing peptone 8.	73
10	"	Flesh (400 g.), H_2O (400 ml.), lingcod stomach (80 g.) and 5N HC1 (20 ml. initially and 10 ml. after 1 day) were digested 20 days. Initial pH 2.0, final pH 4.0.	85
11	"	Flesh leached 1 day was digested under conditions similar to those used in preparing peptone 10.	87

Peptone No.	Fish used	Method of Preparation	Yield %
12	Gray cod (*Gadus macrocephalus*)	Flesh (500 g.) and 2N H_2SO_4 (500 ml.) were hydrolyzed 6 hrs. at 120°C. The pH of the filtrate was adjusted to 7.5 with 850 g. of Amberlite anion-exchange resin IR4B before processing.	54
13	"	Flesh (500 g.) and 2N NaOH (500 ml.) hydrolyzed 6 hrs. at 120°C. The pH of the filtrate was adjusted to 7.2 with 500 g. of Amberlite cation-exchange resin IR 100H which had been adjusted to the hydrogen cycle.	62
14	Lemon sole (*Parophyrys vetulus*)	Flesh (350 g.) and 2N HCl (350 ml.) hydrolyzed 6 hrs. at 120°C. The filtrate was adjusted to pH 7.2 with 325 g of Amberlite anion-exchange resin IR4B.	58
15	Lingcod	Flesh leached 2 days (430 g.) and 3.5N H_2SO_4 (430 ml.) hydrolyzed 6 hrs. at 120°C. The sulphate was removed by addition of $Ca(OH)_2$ (65 g.) and the filtrate (pH 7.4) processed.	78

Peptones 16, 17 and 18 were respectively Bacto Peptone, Bacto Tryptose and Bacto Tryptone.

It will be observed that the yields of peptone differed markedly, probably depending on variations in such factors as the amount of enzyme preparation used, its activity, and the length of hydrolysis period. In general the yields were considerably higher than those reported by Hook and Fabian (1943) who dialysed their flesh hydrolysates in cellophane before drying them. Leaching of the flesh before hydrolysis did not appreciably affect the yield as calculated on the dry weight of the starting material (preparations 8 to 11) nor did it visibly affect the colour of enzyme-hydrolysed preparations. However, where acid hydrolysis was employed for a sufficient time to give at least a 50% yield (preparations 12, 14 and 15), the products were dark brown in colour irrespective of whether the flesh used had been leached or not (table I). Fish flesh contains considerable amounts of glycogen (Macleod and Simpson 1927, Sharp 1934) and this on acid hydrolysis could yield glucose which might react with the amino groups of proteins or their degradation products to yield dark coloured compounds (Maillard reaction). Glycogen is resistant to alkaline hydrolysis, and this method of preparation yielded a light-coloured product (Peptone 13).

No detailed chemical analyses of the peptones were made, though a few of their properties including nitrogen content, pH of solutions, solubilities and colour are given in table I. In general their nitrogen content compares favourably with that given for certain commercial preparations (Difco 1943, Hook and Fabian 1943). Previous work (Deas and Tarr 1947, and submitted for publication 1949) showed that fish flesh hydrolysed for 16 days employing fish pyloric caeca enzymes yielded solutions which contained considerable amounts of peptones, sub-peptones and smaller residues, and it can therefore be inferred that fish peptones prepared by drying such hydrolysates will contain a somewhat

TABLE I. Certain characteristics of the peptones investigated.

No.	Nitrogen content %	Colour	pH of a 10% solution	Appearance of a 10% aqueous solution at room temperature
1	14.7	Light brown	6.60	Clear, light brown.
2	14.0	" "	5.65	" " "
3	14.5	" "	6.55	" " "
4	13.9	" "	6.20	Very cloudy light brown, heavy flocculent precipitate.
5	14.4	" "	7.10	Clear, pale brown to yellow.
6	14.0	" "	6.50	" " " "
7	13.1	" "	6.70	" " " "
8	12.9	" "	6.50	" light brown.
9	13.7	" "	6.40	" pale brown to yellow.
10	12.8	" "	6.65	" " " " "
11	13.5	" "	6.50	" " " " "
12	13.7	Dark brown	6.30	" dark brown.
13	12.9	Light brown.	7.70	Faint cloudiness, pale brown to yellow.
14	13.4	Dark brown	6.25	Clear, dark brown.
15	13.0	" "	6.70	Moderately cloudy, dark brown.
16	15.2	Light brown	6.85	Clear, pale brown to yellow.
17	12.9	" "	7.10	Very cloudy, not all in solution, light brown.
18	12.5	" "	6.95	" " " " " " " "

similar mixture of protein degradation products. Commercial peptones normally contain similar mixed protein breakdown products, and, in addition, considerable quantities of proteose nitrogen, which indicates that less severe hydrolysis had been used in their preparation (Difco 1943, Hook and Fabian 1943). The methods of preparation of the various fish peptones were designed to yield products of relatively low inorganic salt content, for commercial peptones do not usually contain high concentrations of these (Difco 1943).

Superficially at least there was no marked difference in appearance or odour of the fish peptones and commercial products which may be prepared from animal flesh or organs, casein and, possibly, vegetable proteins (Hook and Fabian 1943). The value of fish peptones for specialized bacteriological techniques, such as preparation of toxins, has yet to be determined. The following section shows that fish peptones form excellent growth substrates for certain fastidious bacteria.

GROWTH OF BACTERIA IN PEPTONE BROTH MEDIA

Bacto tryptose phosphate broth and agar media (Difco 1943) containing 10% of added sheep blood were employed for initial cultivation of the haemolytic streptococci. For comparison of the ability of the various peptones to support bacterial growth the usual Tryptose phosphate glucose broth formula (Difco 1943), in which the experimental peptones replaced Tryptose, was employed. The media were adjusted to pH 7.3 to 7.35, 2.5 ml. placed in 13 × 160 mm. tubes and sterilized at 120°C. for 20 minutes. The tubes of broth media were placed in a boiling water bath for a short time and cooled just prior to inoculation.

Inoculations and transfers were made with a 1-mm. diameter platinum loop. All cultures were incubated at 38°C. In the experiments with strains of *Clostridium botulinum* anaerobic conditions were maintained by the chromium sulphuric acid technique of Rosenthal (1937) as modified by Mueller (1941).

STREPTOCOCCUS HAEMOLYTICUS.

Representatives of this genus were selected because many are rather fastidious with respect to their nutrient requirements especially when freshly isolated and transferred from blood-containing media. Lyophilized cultures of the following groups were obtained: A type III, A type IV, B_1, C_1P, D and E. A small amount of each of the desiccated cultures was emulsified in Tryptose phosphate glucose broth using a Pasteur pipette, and some of the resulting suspension was spread over the surface of a blood agar plate. After incubation several isolated colonies were selected from each plate and inoculated into tubes of blood Tryptose phosphate glucose broth. After incubating for one day these blood broth cultures were stored for a week at 0°C. during which time they were used for inoculating the experimental tubes of peptone phosphate glucose broth media. Two separate experiments were carried out in which 18 different broth media prepared from each of the peptones were inoculated with the six haemolytic streptococci under investigation. Growth was observed after 5 hours and thereafter after one- and two-day incubation periods. Where positive growth occurred in these first transfers from blood broth, a second tube of identical medium was inoculated, and, when growth occurred after this second transfer, a third transfer was made. In all instances it was found that where growth occurred after the second transfer in peptone broth a third transfer in identical medium was followed by growth of approximately the same intensity. Since only a very few minor differences were observed in the two identical experiments, the results obtained in only one of them have been recorded (table II). Not all the cultures grew equally well and for the sake of brevity only results obtained with the three cultures which appeared most fastidious, namely, C_1P, A III and A IV, have been recorded. Since no growth was obtained with any culture except B_1 in peptones 12 to 15 these peptones have not been included in the table. In general, fish peptones prepared by tryptic or peptic hydrolysis of fish flesh (No. 1, 3 and 5 to 11) proved most satisfactory. Peptones prepared from the same fish by peptic and tryptic hydrolysis of both leached and unleached flesh (preparations 8 to 11) supported growth about equally well, indicating that all four procedures are satisfactory for peptone preparation. The three commercial peptones were, in general, not as satisfactory as the enzyme-hydrolysed fish peptones in supporting growth of the haemolytic streptococci. Hydrolysis of fish flesh with H_2SO_4 followed by neutralization with $Ba(OH)_2$, $Ca(OH)_2$ or an anion exchange resin (peptones 4, 12 and 15), or with HCl or NaOH followed by neutralization with anion and cation exchange resins respectively (peptones 13 and 14), yielded products which were uniformly unsatisfactory in promoting growth of the test organisms. Peptone 2, prepared by HCl hydrolysis of the non-digestible residue from an enzyme hydrolysate of fish flesh and neutralization of the reaction

TABLE II. Influence of peptone source on growth of *Streptococcus haemlyticus* cultures.

Peptone No.	A III				A IV				C_1P			
	Sub-culture Number				Sub-culture Number				Sub-culture Number			
	1			2	1			2	1			2
	5 Hours	1 Day	2 Days	2 Days	5 Hours	1 Day	2 Days	2 Days	5 Hours	1 Day	2 Days	2 Days
1	−	++	++	−	−	++	++	++	−	++	++	−
2	−	+	+	+	−	+	+	−	−	−	−	
3	−	++	++	++	−	++	++	−	−	++	++	++
4	−	−	−		−	−	−		−	−	−	
5	−	++	++	++	−	++	++	++	−	−	−	
6	−	++	++	−	−	++	++	++	−	++	++	−
7	−	+	++	++	−	++	++	++	−	++	++	−
8	−	++	++	−	−	++	++	++	−	++	++	−
9	−	++	++	++	−	++	++	++	−	++	++	−
10	−	+	++	++	−	++	++	++	−	++	++	−
11	−	+	++	++	−	++	++	++	−	++	++	−
16	−	−	−		−	++	++	−	−	−	−	
17	−	−	−		−	++	++	−	−	−	−	
18	−	+	+	−	−	+	++	−	−	+	++	−

− No visible growth, + feeble growth, ++ good growth.

mixture to pH 6.5 (and not to pH 7.0 or 7.2) with an anion exchange resin, supported growth of the test organisms moderately well. This indicates that one means of obtaining a good yield of peptone from fish flesh would be to carry out preliminary enzyme hydrolysis followed by separate acid hydrolysis of the undigested residue and then to combine the filtrates before concentrating them to dryness.

It was thought that the poor nutritive value of certain of the peptones might have been in part due to the destruction of B vitamins during preparation. In order to test this possibility an experiment identical to the above was run except that, before sterilizing the media, the following amounts of vitamins were added per tube of broth: niacin and calcium pantothenate, 125 μg.; pyridoxin and para aminobenzoic acid, 25 μg.; and biotin and folic acid, 1.2 μg. The results showed that the addition of these vitamins facilitated the growth of the haemolytic streptococci on only a few of the media; the following differences being noted in comparison with the foregoing experiment without added vitamins. Peptone 12 slightly improved growth of cultures B_1 and D; peptone 13, slow growth of culture D; peptone 14, good growth of cultures B_1 and D; peptone 15, good growth of cultures B_1 and D, moderate growth of cultures B_1 and E and slight growth of culture A III; peptone 16, transient growth of culture E. Thus with peptones 12, 13 and 14, in which ion-exchange resins had been used to adjust the pH of the hydrolysed solutions to about 7.0 or 7.2, the addition of B vitamins to

the media rendered possible growth of certain of the organisms and improved that of others. The addition to these vitamins to the medium made from peptone 15 in which sulphate had been removed by $Ca(OH)_2$ improved its availability to several of the test organisms. It is evident that all peptones made by enzyme hydrolysis of fish flesh contained adequate quantities of the above B vitamins, since their addition did not facilitate growth in media prepared from such peptones. There is little doubt that such peptones would also contain strepogenin and vitamin B_{12}.

CLOSTRIDIUM BOTULINUM.

Two cultures of type A and one each of type B and E were studied. Washed spores of the organisms were obtained as follows. Centrifuge tubes containing 50 ml. of glucose-free Bacto tryptose phosphate broth were inoculated and incubated for 4 days. The broth cultures were heated for 10 minutes at 80°C. to destroy vegetative cells, and they were then centrifuged and the spores suspended in, and washed twice with, successive 25-ml. portions of water. The washed spores were suspended in 50-ml. of water and stored at O°C. for 7 days during which the suspensions were used to inoculate the peptone media.

A preliminary experiment in which broth tubes of the 18 peptones under investigation were inoculated with each of the 4 *C. botulinum* cultures showed that, with the single exception of type E, all grew quite well on all the media. The results of an experiment in which the type E culture was inoculated into the various peptone media and the cultures incubated for 4 days are given in table III. In this experiment it was found that where growth occurred after a second

TABLE III. Influence of peptone source on growth of type E *Clostridium botulinum*.

Peptone No.	Growth after first transfer	Growth after second transfer
1, 3, 5-11 and 16-18	+++	+++
2 and 13	++	++
4	+	+
12 and 14	+	−
15	++	−

− no visible growth, + feeble growth, ++ moderate growth, +++ good growth.

transfer in one of the test media growth of similar intensity resulted after a third transfer. Excellent growth occurred in all fish peptones prepared by enzyme hydrolysis and also in the three commercial preparations. In media prepared from peptones 2, 4 and 12 to 15, in which hydrolysis had been carried out by acid or alkali followed by neutralization of the reaction mixtures by different methods, growth was either less satisfactory than in the enzyme-hydrolysed preparations, or poor and transient.

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The Effect of pH on the Enzymatic Reduction of Trimethylamine oxide

By C. H. Castell and J. M. Snow
Atlantic Fisheries Experimental Station, Halifax, N.S.

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ABSTRACT

The reduction of trimethylamine oxide by heavy suspensions of washed cells of a number of species was greatly affected by the pH of the substrate, the more acid the reaction the slower was the reduction.

The muscles of most of our marine fish lie within a range of pH 6.0 to 7.5. We are interested particularly in the effect of pH within this range on the enzymes which are responsible for fish spoilage.

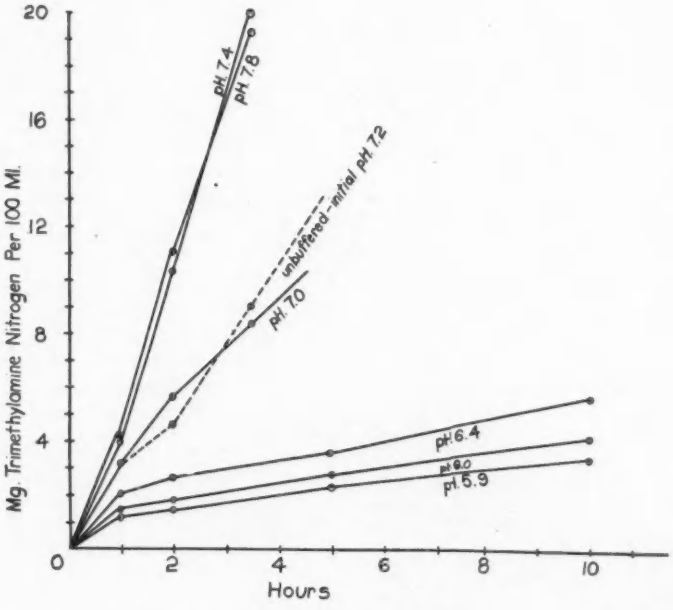

Figure 1. The effect of pH on the production of trimethylamine by heavy suspensions of washed bacterial cells.

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By using very heavy suspensions of washed bacterial cells as the source of the enzymes we have observed that the trimethylamine oxide reducing system is significantly affected by differences in pH between 5.9 and 7.8 (Figure 1).

The culture used was *Ps. putrefaciens*, isolated from fish. The substrate consisted of trimethylamine oxide, sodium acetate as the hydrogen donor and sodium hydroxide-phosphate buffers. These were incubated in Thunberg tubes at 30°C. As the amine is highly volatile in alkaline solutions and is changed to a non-volatile salt in acid solutions, and because samples to be tested were removed periodically, the tubes were made air tight but were not evacuated.

Similar tests have been made using cells of *E. coli*, *A. Aerogenes*, *S. marcescens*, *P. vulgaris* and other organisms isolated from fresh and salted fish. There was some variation in the pH ranges with the different species tested. But in every case as the pH was made more acid from a neutral reaction, the enzyme activity became less; and at pH 6.0 and below it became almost insignificant.